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A STUDY ON

**Proteinbound
Sulfhydryl Groups
in Pulmonary
Cytodiagnosis**

By Lars-Gösta Wiman

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A STUDY ON

Proteinbound Sulfhydryl
Groups in
Pulmonary Cytodiagnosis

*and their significance in cancer diagnosis
compared with the Papanicolaou technique and
acridine-orange fluorescence microscopy*

BY

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CONTENTS

INTRODUCTION	7
CHAPTER I. <i>Review of the literature</i>	9
1. Morphological pulmonary cytodiagnosis	9
2. Fluorescence microscopy and acridine-orange	12
3. Bio- and histochemistry of sulfhydryl groups	14
4. Methods for the demonstration of sulfhydryl groups	18
CHAPTER II. <i>Material</i>	21
CHAPTER III. <i>Technique of collecting specimens</i>	31
1. Sputum	31
2. Bronchial secretions	33
CHAPTER IV. <i>Staining Techniques</i>	35
1. Staining for the demonstration of proteinbound sulfhydryl groups in cytological smears (the SH-method)	35
2. Staining by acridine-orange for fluorescence microscopy (the AO- method)	38
3. Staining according to the Papanicolaou technique	40
CHAPTER V. <i>The appearance of cells in sputum and bronchial secretions</i> .	41
1. The sulfhydryl(SH) method	41
2. Microphotometric transmission measurements of exfoliated cells stained by the SH-method	45
3. The acridine-orange(AO) method	47
4. The Papanicolaou method	51
5. Classification of the exfoliated cells	51
SH-reaction	54
CHAPTER VI. <i>Results of cytological assessment</i>	55
1. The sulfhydryl(SH) method	55
A. Sputum	55
B. Results of cytological examination after restaining of sputum smears by the SH-method	61
C. Bronchial secretions	62
2. The acridine-orange(AO) method	65
A. Sputum	65
B. Bronchial secretions	68

3. The Papanicolaou method	69
A. Sputum	69
B. Bronchial secretions	72
4. Various combinations of the sulfhydryl(SH), acridine-orange(AO) and Papanicolaou methods	75
DISCUSSION	81
1. Exfoliation	81
2. Specimen collection	82
3. Staining methods and results	85
A. The acridine-orange(AO) method	85
B. The sulfhydryl(SH) method	87
C. The Papanicolaou method	91
SUMMARY	93
ZUSAMMENFASSUNG	95
RESUMEN	98
RÉSUMÉ	100
ACKNOWLEDGEMENTS	103
APPENDIX. <i>Case reports</i>	105
REFERENCES	117

INTRODUCTION

In many forms of cancer good results of treatment are achieved by surgery, radiotherapy and chemotherapy. In spite of a considerable technical progress in these fields the five year survival in bronchogenic carcinoma does not usually exceed 5-10%. A contributory cause of this bad prognosis is, above all, that the diagnosis is obtained too late in the course of the disease. For this reason an early cancer diagnosis should always be an essential aim, independent of the therapeutic resources, and the day an effective treatment against cancer is introduced, we should be diagnostically prepared.

In primary bronchogenic carcinoma, a tentative diagnosis may be obtained by a carefully elicited case history, physical examination, photofluorography, radiography and other kinds of clinical and laboratory investigations. A final and definite cancer diagnosis, however, can be obtained only by histopathological examination of biopsy material secured from the primary tumor at bronchoscopy and thoracotomy or from an accessible metastasis. In this connection pulmonary exfoliative cytodiagnosis with microscopical examination of sputum, bronchial secretions and pleural fluid occupies an intermediate position between clinical and histopathological diagnosis. This method cannot usually be equated with histopathological examination, but may be of special value, for instance, in mass surveys or when biopsy for anatomical or other reasons is impossible to perform. Cytological examination can also be used on considerably wider indications than can biopsy and without undue risks or discomfort to the patient.

The methods of exfoliative cytodiagnosis used for the detection of bronchogenic carcinoma vary in regard to the technical procedure and are based upon different theoretical considerations. Smears or sections can be fixed or stained in various ways, and light field microscopy as well as phase contrast, interference and fluorescence microscopy may be used. A great number of methods have been tried out for the purpose of establishing the morphological details characteristic of malignancy. These conventional, morphological methods, however, require a long training and a large experience of microscopical examination of cells.

In the 1950's serious attempts were first undertaken in the field of clinical cytology using cytochemical staining methods to obtain information of different kinds other than morphological, for instance, the increased nucleic acid content of certain cancer cells, using acridine-orange staining and fluorescence microscopy.

On account of a correlation between protein synthesis, cellular growth and sulfhydryl(SH) groups many biochemical and histochemical methods were introduced to demonstrate SH-groups, which were of great importance in cancer research. As a link in this development it was natural to investigate the presence of SH-groups in exfoliated cells, partly with the purpose of testing the possibilities for cytodiagnosis of malignancy. The results of these preliminary investigations on cytological material from the respiratory tract (Wiman 1959, 1960, 1962, 1963) initiated further investigations.

For these reasons the purpose of this work was to study the presence of proteinbound SH-groups in exfoliated cells found in sputum and bronchial secretions secured at bronchoscopy, and to see to what extent this cytochemical method could be used in routine cancer diagnosis. A further aim was to compare these empirical findings of proteinbound SH-groups with the results of staining by the acridine-orange fluorescence method and the Papanicolaou technique before the final correlation to histopathological and clinical diagnoses.

REVIEW OF THE LITERATURE

I. MORPHOLOGICAL PULMONARY CYTODIAGNOSIS

That the quantity of the expectorate, its consistency, appearance, admixture of blood, duration, etc. could be of importance in the diagnosis of lung cancer was already emphasized in 1842 by Stokes, who pointed out the resemblance of red currant jelly to sputum from certain cancer patients. In primary carcinoma of the lung, hemoptyses, or blood tinged sputa, were present in at least two thirds of all cases. That certain expectorated lumps or particles are connected with lung cancer was recognized and described by several authors, e.g. Walshe (1851), Hampeln (1876) and others, cited by Wandall and Grunze.

With the development of cellular pathology and technical progress in the construction of the microscope, the composition of several human body fluids could be more closely studied. In 1851 Walshe wrote that with the help of the microscope cancer cells could be identified in sputum where nothing suspicious would appear under the naked eye. Microscopically, Beale (1860) demonstrated neoplastic cells in sputum from a patient with carcinoma of the pharynx and Hampeln (1876) in sputum from a patient with an osteosarcoma and pulmonary metastases. Cancer cells in sputum from a case of primary lung cancer were first recognized by Hampeln (1887) who gave a detailed description of their size, form and appearance. This case, as well as Beale's, was illustrated with drawings of cells showing morphological signs, which are still characteristic of squamous cell carcinoma. In these microscopical examinations of sputum only unstained smears appear to have been used.

From 1890 onwards frequent reports appear in the literature of tumor cells in sputum which were found in smears or histological sections upon using simple methods of fixation and staining. These works have been exhaustively reviewed by Wandall (1944) and Grunze (1960). The interpretation of the cell picture varied widely. Betschart and Hampeln attached to solitary atypical cells even a great diagnostic value, while other authors demanded cell clusters or macroscopically recognizable tumor fragments, which could be investigated by histopathological techniques, for a satisfactory diagnosis of malignancy. The practical use of this method, however, was emphatically denied by Krampf & Sauerbruch (1935), and others.

Compilations of large materials to show the value of cell examinations in the diagnosis of cancer were first published by Hampeln (1918). In 25 patients with primary carcinoma of the lung, sputum examinations were performed using unstained smear preparations. Cancer cells could be demonstrated in 13 patients, that is a correct positive cancer diagnosis of 52%.

With the development of thoracic surgery new therapeutical possibilities were opened, and the importance of an early diagnosis was evident. One possibility of attaining this goal was to be found in examining the cells of sputa, and of secretions aspirated through the bronchoscope. A new cytological technique for this purpose was introduced by Dudgeon & Wrigley (1935) who immediately after collecting the specimen immersed the smear preparations of sputum in a fixative composed of ethyl alcohol, mercuric chloride and glacial acetic acid. Thus shrinking and volume reduction of the cells following air drying was avoided. The staining was performed with hematoxylin and eosin. This wet film technique was developed on the basis of a method according to Dudgeon & Patrick (1927) and Dudgeon & Barret (1934) for quick diagnosis of surgical specimens or biopsies taken during operations with regard to the presence of a malignant tumor. Using the method described above Dudgeon & Wrigley (1935) were able to demonstrate malignant cells in sputum in 74% of 34 patients with lung cancer. Using the same method Barret (1938) recognized tumor cells in sputum in 68% of 100 cancer patients. Edwards (1938), considered the method to be of great value in the diagnosis of cancer, and using sputum examinations combined with bronchoscopical biopsy he succeeded in reaching a positive cancer diagnosis in 90% of patients.

Gowar (1943) identified tumor cells in sputum in 64% of 56 cases of lung cancer, which, in this material, made more operative interventions possible. Of greatest importance was the work of Wandall (1944) in which neoplastic cells were identified in 84 of 100 patients with primary carcinoma of the lung, and, combined with bronchoscopy, a positive cancer diagnosis was reached in 94%. A modification of Dudgeon & Wrigley's method was used.

Less satisfying results have also been reported. Using cytological sputum examination Craver (1940) found only 2 positive cases out of 175 patients with lung cancer, and McGibbon & Baker-Bates (1941) were able to demonstrate cancer cells in but a few advanced cases of 163 patients with pulmonary tumors. Overholt obtained a positive cytologic diagnosis in 66% of patients, but all except one were inoperable or had a positive cancer diagnosis with biopsy at bronchoscopy, and for these reasons the method was considered to be of little practical value. Similar results were obtained by Lecoeur (1942) who did not succeed in demonstrating a single cancer cell in sputum from 52 patients with pulmonary cancer. Björkman (1943) recognized suspected

TABLE 1. *Results of cytological examination of sputum and bronchial secretions in bronchogenic carcinoma by different authors*

Author	Year	Material	No. of cancer cases	Correct positive diagnoses, per cent of cancer cases	False positive diagnoses, per cent of overall cases
Hampeln	1918	Sputum	25	52	
Dudgeon & Wrigley	1935	Sputum	38	68	1.7
Wandall	1944	Sputum	100	84	3.1
Papanicolaou	1946	Sputum	31	71	0
Liebow <i>et al.</i>	1948	Sputum	49	43	3.8
Kucsko & Portele	1949	Sputum	94	87.2	
Farber <i>et al.</i>	1950	Sputum, 1 specimen	196	57	< 1
		Sputum, 5 specimens	130	90	
		Bronchial secretions	113	35	< 1
Kahlau	1951	Sputum	71	76	4.8
Heckner	1952	Sputum	78	10	
		Bronchial secretions	78	20	
Hartmann <i>et al.</i>	1953	Bronchial secretions	160	67.5	2.4
Hjelt	1953	Sputum	171	70.7	2.6
		Bronchial secretions	39	79.5	3.3
Rome & Olson	1957	Sputum	172	93	
		Bronchial secretions	129	79.8	
		Sputum and bronchial secretions	266	76.3	< 1
Umiker	1960	Sputum, 1 specimen	94	30	
		Sputum, 3 specimens	94	71	
		Sputum, 5 specimens	94	83	
		Sputum, more than 5 specimens	94	96	< 1
		Bronchial secretions	71	40	
Russel <i>et al.</i>	1963	Sputum, 2 specimens	61	23	
		Sputum, more than 10 specimens	95	77	< 1
Wiman	1963	Sputum, 1 specimen	73	42	
		Sputum, 5 specimens	20	87	
		Bronchial secretions	62	42	

cancer cells in sputum from one of 17 lung cancer patients, and Björk's investigations (1947) revealed positive sputum specimens in only 4.7% of 345 patients with the clinical diagnosis of bronchogenic carcinoma.

After many years of study concerning the changes of the vaginal epithelium under hormonal influence Papanicolaou (1942) published his staining method for vaginal smears, which was later to be of the greatest importance in the

development and expansion of exfoliative cytology. Papanicolaou & Traut (1943), in a monograph on this subject showed the possibilities of improving the diagnosis of uterine cancer by using this method. Applied to smears of sputum and bronchial secretions Papanicolaou (1946) obtained a result of 66% correct positive cancer diagnosis. A series of papers now appeared in which different authors, using Papanicolaou's morphological staining, stated that the possibilities of a positive diagnosis usually were 50–95% in patients with carcinoma of the lung. The results, however, were dependent upon the composition of the materials. Sputum as well as bronchial secretions were usually used together in combination (Table 1).

2. FLUORESCENCE MICROSCOPY AND ACRIDINE-ORANGE

Fluorescence microscopy is capable of raising the sensitivity of any histochemical method whose final product in the tissues is fluorescent (Pearse, 1960). This great sensitivity, which is the main advantage of the method, is coupled with the possibility of creating a high contrast and with the very low concentrations of the excitable material necessary to provide visible fluorescence. By adding minimal quantities of the fluorescing substance the method can be used for studying living cells. Fluorescence microscopy has gained great use especially in detecting small quantities of various materials in tissues. This was employed for substances with primary fluorescence, such as, tryptophan by Jancsó (1932), para-amino-salicylic acid (PAS) by Alin & Helander (1948), dihydrostreptomycin and tetracyclines by Abello (1954) and quinacrine (atebrin) by Mustakallio (1954). The administration of tetracycline excited a yellow fluorescence in animal tissue as shown by Helander & Böttiger (1953), Böttiger (1955) and Weissberg (1959). The fluorescence, however, disappeared after a short while, with the exception of bone, according to Milch, Rall & Tobie (1957), Hulth & Olerud (1961), and tumor tissue according to Rall, Loo, Lane & Kelly (1957), Loo, Titus & Rall (1957) and Owen (1961). For diagnostic purposes this was used in different forms of cancer, e.g. by McLeay (1958), Klinger & Katz (1961) Berk & Kantor (1962), Berk (1963) and in exfoliative pulmonary cytology by Wiman (1963).

Substances which do not primarily fluoresce can be combined in many ways with a fluorescing compound through fluorochromation, and hence be demonstrated histochemically. Thus, one of the largest fields of fluorescence microscopy uses the fluorescent antibody technique, in which the reactions following injections of foreign protein antigens can be studied (Coons, Creech & Jones, 1941, and others).

One of the most widely used fluorochromes is acridine-orange (AO) introduced into histochemical work by Bukatsch & Haitinger (1940) and Strugger

(1940). Gössner (1949) and von Bertalanffy & Bickis (1956) were able to show the connection of AO to ribonucleic acid, and a varying degree of polymerization of the cell nucleic acid was thought to explain the green or red AO-fluorescence (Schümmelfeder, Ebschner & Krogh, 1957).

Fluorescence microscopy was introduced in exfoliative cytology by Friedman (1950) who stained vaginal smears with a great many fluorescing compounds in varying concentrations and at different pH. The best results were obtained using a combination of neutral berberin sulfate, acid fuchsin and acridine yellow in a buffer solution of pH 8 after fixation in equal parts of ether and ethyl alcohol. Malignant cells fluoresced with a marked, deep orange-red color of the nucleolus and with a varying degree of orange or red-orange of the cytoplasm. At the same time the cells displayed the same morphology and criteria of malignancy as observed with the Papanicolaou technique. The author mentioned that this method was also tried on specimens of sputum, ascitic and pleural fluid for diagnosis of malignant cells.

L. von Bertalanffy, Masin & Masin (1956 and 1958) critically studied the Friedman method and performed other experiments using fluorescence microscopical techniques on malignant cells for gynecological cancer diagnosis. They found most of them as equally time-consuming as the Papanicolaou method or affected by other disadvantages. Based on the work of L. von Bertalanffy & Bickis mentioned above, they recommended fluorochromation with acridine-orange (AO). The authors described a quick and simple procedure suitable for vaginal and cervical smears in which malignant cells could easily be detected. *After the AO-staining the cancer cells appeared with a strong, brilliant red-orange fluorescence of the cytoplasm and nucleolus, thus indicating a high concentration of ribonucleic acid (RNA) and an intense yellow staining of the nuclear chromatin as a manifestation of the increased content of desoxyribonucleic acid (DNA).* Preliminary studies with this method were performed on material from tumors of the stomach, rectum, lung and the breast.

Umiker, Pickle & Waite (1959) used a modification of the AO-method on smears of sputum, bronchial secretions, ascitic and pleural fluids, urine, stomach washings and secretions from irradiated oral tumors. In these studies the nuclei of the malignant cells appeared green (low DNA content) or yellow to yellow-white (high DNA content), while cytoplasm and nucleoli were stained intensely orange or red (high RNA content). Malignant cells with a low RNA content, however, exhibited a green cytoplasmic fluorescence. With the AO-technique keratin could not be demonstrated and the squamous epithelial character of malignant cells had to be decided upon by morphological criteria. The best result was obtained from pleural and ascitic fluid specimens with a 100% of correct positive cancer diagnosis (8 cases)

and sputum with 87% positive findings (62 cases). The total material showed a correct cytological cancer diagnosis in 83% of cases. A decided disadvantage of the method was the high percentage (16.2%) of false positive cancer diagnoses. Staining with the Papanicolaou method showed a higher sensitivity. Thus both advantages and disadvantages were connected with the AO-technique.

Dart & Turner (1959) changed the original AO-method and used buffer solutions of pH 3.8. They examined 5,000 vaginal and cervical smears and 500 specimens from sputum, bronchial secretions, pleural, ascitic and lymphatic fluids, mammary secretions and urine. For differentiation of DNA and RNA, CaCl_2 was substituted by McIlwain's buffer solution with a pH of 3.8. The results equalled those obtained with the Papanicolaou method, but the AO-staining was considered to be quicker and more easily performed.

F. D. Bertalanffy (1960) reported further work and experience with the AO-method using material of sputum, bronchial secretions, pleural and ascitic effusions, stomach washings, vaginal and cervical secretions, urine, blood from leukemic patients, bone marrow, aspirates from cysts, knee joints, inguinal lymph glands, liver, brain, melanomas, sarcomas, experimental tumors and other secretions. In all cases malignant cells were identified through their striking orange or red fluorescence.

L. v. Bertalanffy & F. D. Bertalanffy (1960) made detailed descriptions of normal and malignant cells in a special study of secretions from the respiratory tract.

3. BIO- AND HISTOCHEMISTRY OF SULFHYDRYL GROUPS

As long as knowledge is still lacking concerning specific differences between normal and malignant growth processes great attention has been devoted to cell protein synthesis and related problems in biochemical cancer research. Cell division has been observed from biochemical and histochemical points of view in tissue culture, *in vitro*, *in vivo*, animal and clinical experiments, etc. In tumors and normal tissue quantitative differences have been demonstrated in the protein composition of the living material, and therefore the synthesis of these substances has acquired great interest and has been the subject of intensive investigations.

Changes in the protein synthesis of the cells will also be reflected in changes in the number of actively reacting radicals of the compounds participating in the reactions. In this respect the presence of sulfhydryl (SH)-groups has acquired a basic importance on account of its universal distribution in a variety of organisms.

SH-compounds in living cells are first mentioned in scientific literature

by de Rey-Pailhade (1888) who described an SH-containing substance called philothion. The same SH-compound was described by Hopkins (1921) who gave it the present day name of glutathione. Later, the distribution and importance of SH-groups have been discussed in many papers. Thus Barron (1951) described a great number of important enzymes requiring SH-groups for their activity, which, for this reason, could influence cell division, cell growth, cell respiration, keratin formation and many other vital functions and reactions of living organisms. The distribution of SH-groups in the normal tissue of animals and human beings is described by Hopkins (1921), Barnett & Seligman (1952), Barnett (1953), Bahr & Moberger (1958), Ellman (1959) and Calcutt & Doxey (1962). Glutathione has the widest distribution of all SH-compounds present in virus, bacteria, plants, animals and human beings and plays a prominent role in many oxidation-reduction systems according to the reaction:



In these reactions, glutathione is of great importance for the respiration, metabolism, division and growth of cells (Barron, 1953; Mazia, 1954, 1961).

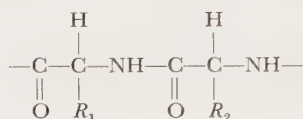
In a series of remarkable experiments with sea urchin eggs (Rapkine, 1930) found a connection between cell division and SH-groups. The glutathione content of the eggs was estimated before and after fertilization and at intervals after cell division. The concentration of glutathione diminished immediately after fertilization reaching a minimum after 30 minutes. After that a sudden increase was noted with a maximum 45 minutes after fertilization, or immediately before the next cell division. The mitoses ceased after adding HgCl_2 , but started again when cysteine or thioglycol was added. Similar observations were made on other biological material by Voegtlin & Chalkley (1930), Hammet & Wall Smith (1931), Hopkins & Morgan (1943), Neufeld & Mazia (1957) and Sakai & Dan (1959).

In many animal experiments the concentration of glutathione has been studied in tumors, giving partly contradictory results: Voegtlin & Thompson (1926), Görner & Görner (1932), Woodward (1935), Greenstein (1942), Zamecnik & Stephenson (1949), Hirono (1960), Holsti (1960) and others. Some connections between non-protein SH and cell growth were noted, but it is not possible to draw valid conclusions on the fate of glutathione in the development of malignant tumors.

Some authors estimated glutathione in human blood and found an increased concentration in leukemic patients correlated to the white or red blood cell count, viz. Parker & Kracke (1936) and Bichel (1944, 1946). Contopoulus & Andersson (1950) found a higher glutathione content in leukemic than in normal leucocytes. An increase of glutathione in the plasma of cancer

patients over the normal plasma level was, under certain conditions, noted by Schoonover (1935) and Osterberg, Coffey, Barga & Dixon (1938).

In animal tissue SH-groups are present mainly as low molecular compounds, for example, glutathione and cysteine, or bound to proteins. These proteins are built up of bundles of polypeptide chains, which, in their turn are composed of amino acids bound to each other by α -amino (NH_2)- and α -carboxyl (COOH)-groups:



Several radicals: R_1 , R_2 , etc., e.g. SH-groups, are linked to the side chains, which, together with the different amino acids of the actual polypeptides, form a pattern characteristic for each special protein molecule. By the position of the linkage within the molecule the possibilities of the SH-group to react with other compounds can be estimated.

Based on such determinations Barron (1951) differentiates between freely reacting proteinbound SH-groups giving a positive nitroprussid reaction and reacting easily with weak oxidation agents, such as, ferricyanide, iodine benzoate, etc., and other SH-groups giving a negative nitroprussid test and first reacting with more powerful oxidizing agents, such as iodine and mercaptide-building compounds, i.e. *p*-chloro-mercury-benzoic acid (PCMB). Finally, there are SH-groups which are so well protected that denaturing with accompanying depolymerisation of the protein molecule is necessary before they can react with other agents (Neogy, Pascal & Roy, 1961).

According to Mazia (1954) his co-worker Ellis could reproduce in all details Rapkine's experiments, mentioned above, using sea urchin eggs, obtaining the same variations of the glutathione content of the eggs. In addition Ellis made simultaneous biochemical estimates of glutathione and proteinbound SH-groups before and during cell division and he obtained curves which were mirror images of each other.

In a mouse epithelioma, Eisen, Montagna & Chase (1953) found in the deep parakeratotic layer of the Malpighian stratum a very intense staining reaction with the Barnett & Seligman method for histochemical demonstration of proteinbound SH-groups. In the same way Setälä, Äyräpää, Niskanen, Stjernholm and Nyholm (1960) demonstrated histochemically a high concentration of proteinbound SH-groups in the epidermis of rats locally treated with 9,10-dimethyl-1,2-benzanthracene (DMBA) and 20-methyl-cholanthrene (MC).

Using the improved technique for demonstrating histochemically protein-

bound SH-groups performed by Barnett & Seligman (1952) and by Bahr (1957) cytophotometric estimations of proteinbound thiols in HeLa cells (tumor cells in tissue culture) were made by Sandritter & Krygier, 1959. The results obtained were in conformity with Ellis's experiments. Thus, cells in interphase showed a relatively high concentration of SH-groups, about the same in the cytoplasm and the nucleus, but highest of all in the nucleolus. During mitosis the concentration of proteinbound SH-groups increased distinctly in both myxoplasm and chromosomes during pro- and telophase, while a corresponding decrease was noted in meta- and anaphase. These changes were correlated with a varying degree of polymerisation of the cell proteins and their sol or gel state (Mazia, 1955). Any real increase of the total number of SH-groups was, however, not considered to occur during mitosis.

During the last ten years several papers have been published concerning the distribution and concentration of proteinbound SH-groups in tumors of human origin. Barnett (1955), together with Reiner, examined the histochemical occurrence of proteinbound SH-groups in 80 tumors of human origin. They found that benign tumors usually contained a smaller quantity of SH-groups than did malignant tumors, of which those of epithelial origin had the highest SH-concentration in the cells. No decided correlation was observed between SH-concentration and the degree of malignancy regarded from a histopathological point of view. Independently of differences in SH-content between different tumors a high color intensity was obtained in tumor cells compared with corresponding normal cells of homologous tissue. Particularly cell nuclei and nucleoli developed a high concentration of proteinbound SH-groups as shown by the Barnett-Seligman method.

The same histochemical technique was used by Föraker & Wingo (1956b) in a study of 42 basal cell cancers of human origin. They concluded that invasive squamous cell carcinoma developed a histochemical reaction pattern varying with the histopathological differentiation and the SH-concentration rose with increasing intracellular keratinization. Similar results were obtained by Föraker & Wingo (1956a) when investigating proteinbound SH-groups in biopsies of a material derived from gynecological patients consisting of 28 cases of invasive squamous cell carcinoma from the uterine cervix, 3 cases of intraepithelial carcinoma and 57 cases of non-neoplastic changes of the cervix. SH-groups were demonstrated in all epithelial cells, and the staining intensity was almost identical in proliferating cells of the deep layers from the vaginal portio, and from metaplastic epithelium, intraepithelial and invasive carcinoma. SH-groups were also observed in regions showing neoplastic and non-neoplastic keratinization by the Barnett-Seligman method.

More pronounced differences, however, were obtained in the concentration of proteinbound SH-groups in an important study by Bahr & Moberger (1958)

where a modification of Barnett & Seligman's method was used (see page 20). In epithelial tumors, i.e. carcinoma of the breast or the uterine cervix, a high SH-concentration was demonstrated, especially in the outer, well nutriated cell layers of the solid cancer cords with a high content of cell proteins. The same conditions were noted in the infiltration zones of the tumor, which showed a more pronounced staining reaction than did the rest of the tumor of the corresponding homologous tissue from which the tumor originated. Differences in SH-activity were also registered in tumors of various histopathological types. In mesenchymal malignant tumors, i.e. fibro- and myosarcoma, the SH-reaction was very marked but more equally distributed within the malignant tissue. A carcinoma of a low differentiated squamous cell type showed an intense SH-reaction, which was more pronounced than that found in a highly differentiated tumor of the same type. Mucine producing adenocarcinomas, i.e. carcinomas of the intestinal tract, showed a fairly weak SH-reaction. No positive correlation was found between SH-activity and the degree of malignancy. The authors also performed microspectrophotometrical estimations of the color intensity of varying cells, and found a SH-concentration two or three times higher in the peripheral than in the central parts of about 50 solid cancer cords.

Using the Bahr method and an ultramicrospectrograph Caspersson & Révész (1963) performed quantitative cytochemical measurements on mouse ascitic tumor cells. They found an elevated protein sulfhydryl level in a cell line of high radiosensitivity.

A study on the SH-content of epithelial cells of normal and purulent bronchial secretions was performed by Gaini (1962) using an organic mercurial method. Malignant cells, however, were not investigated.

4. METHODS FOR THE DEMONSTRATION OF SULFHYDRYL GROUPS

A great number of methods for the histochemical demonstration of protein-bound SH-groups are to be found in the literature, most of which are described and commented upon by Bahr & Moberger (1958) and Pearse (1960). Usually an original biochemical method has been revised and adapted for histochemical use. This was true for one of the earliest methods where sodium nitroprussid, $\text{Na}[\text{Fe}(\text{CN})_5\text{NO}]$, reacts with the SH-radicle giving a reddish-violet color, the intensity of which is directly proportional to the SH-concentration (Mörner, 1899). The method was introduced into histochemistry by Buffa (1904) to demonstrate compounds with free SH-groups, such as cysteine. It has, however, both theoretical and practical disadvantages.

Several other methods received attention, but they were rejected as either

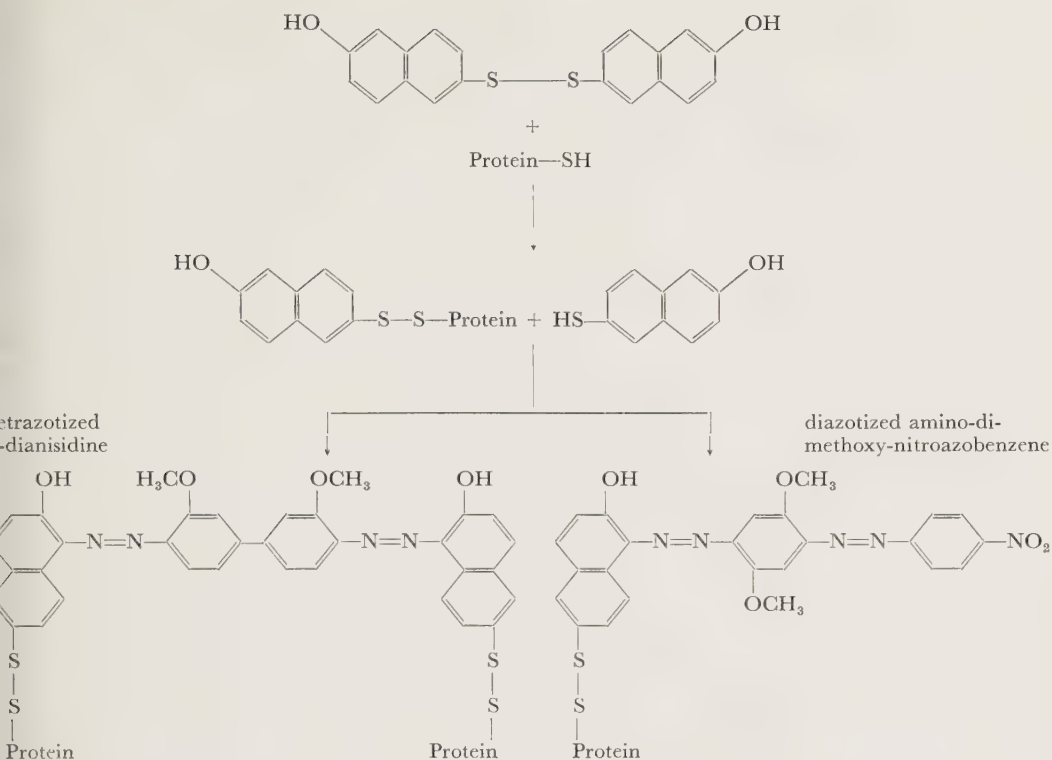


FIG. 1. The DDD-reaction: DDD (2,2'-dihydroxy-6,6'-dinaphthyl disulfide) is coupled to a proteinbound SH-group; one half of the molecule is split off and dissolved out. Left: reaction with *o*-dianisidine (dicoupling). Right: reaction with Fast Black Salt K (monocoupling) (Bahr, 1957).

too unspecific or complicated, or were for other reasons considered less suitable for histochemical use (Sullivan, 1926, Anson, 1941, Chévrement & Frédéric, 1943, Bennett & Yphantis, 1948, Danielli, 1950, Dempsey, Singer & Wislocki, 1950).

The most specific method for the histochemical demonstration of protein-bound SH-groups was introduced by Barnett & Seligman (1952), and modified by Bahr (1957).

A substance containing a disulfide linkage, 2,2'-dihydroxy-6,6'-dinaphthyl disulfide (DDD), was synthesized by Barnett & Seligman. DDD specifically oxidizes SH-groups bound to proteins, and two colorless compounds are formed, protein-naphthyl disulfide and naphthyl mercaptan (see Fig. 1). Excess of DDD and naphthyl mercaptan are insoluble in organic solvents and are removed in a series of alcohols and ether. A diazonium salt, Fast Blue B or tetrazotized *o*-dianisidine, reacts with the protein-naphthyl disulfide forming

a colored substance. In the tissues a blue color indicates a high and a red color a low concentration of proteinbound SH-groups. To show the specificity of the reaction, Barnett & Seligman performed a variety of suitable tests.

As substitutes for DDD Seligman, Tsou & Barnett (1955) examined other naphthol compounds, e.g. 4-hydroxy-1-naphthyl-N-maleimide(= HN.M), 4-hydroxy-1-naphthyl mercuric acetate and 4-(*n*-iodoacetyl)-amino-1-naphthol, in combination with Fast Blue B. None of these compounds, however, offered any further advantages.

Price & Campbell (1957) used these reactions in paper chromatography and found a reaction product after HN.M and Fast Blue B showing an intense red fluorescence at $366\text{ m}\mu$ in ultra-violet (UV) light, which could be used for a histochemical UV-method (Pearse, 1960).

To test the DDD-method Bahr (1957) performed a series of model experiments with diazotate of 4-amino-3,6-dimethyl-4-nitroazobenzene (Fast Black Salt K or Echtschwarzsatz K) instead of Barnett & Seligman's Fast Blue B. In this way a single shade of deep blue was obtained with varying color intensity depending upon the concentration of the SH-groups bound to protein. Using back titration with DDD and ovalbumin Bahr was able to show that a *linear relation existed between color intensity and the number of accessible SH-groups*. The single blue color with a varying intensity now made it possible to perform quantitative SH-determinations on the basis of absorption measurements. Results of such investigations were published by Bahr (1957), Bahr & Moberger (1957, 1958), Sandritter & Krygier (1959) and Caspersson & Révész (1963). Principal differences between the staining techniques used by Barnett & Seligman and Bahr are demonstrated in Fig. 1.

MATERIAL

Cytological examination of sputum and bronchial secretions was performed by three methods, namely, staining of smears for the demonstration of protein-bound sulfhydryl (SH) groups, with acridine-orange (AO) and fluorescence microscopy according to L. von Bertalanffy, Masin & Masin and staining by the Papanicolaou technique. The investigations were made over a period of four years, 1958–1961, the specimens being collected from various hospitals in Uppsala, Gävle and Enköping. The comprehension of the material is shown in Tables 2 and 3.

All specimens containing cellular material from the upper respiratory tract only were rejected as not representative for bronchi and alveoli. Patients who did not succeed in producing a specimen representative for the lower respiratory tract either spontaneously or after artificial induction were not included in the material.

Every patient was admitted to a hospital for investigation of symptoms of respiratory tract diseases or uncertain findings at photofluorographical or X-ray examination. The most common indications leading to cytologic investigation of sputum or bronchial secretions were persistent cough of extensive duration with or without expectoration, hemoptysis, dyspnoea, pain in the chest, uncertain fever conditions, etc. The symptoms often appeared in association with X-ray changes of varying distribution, such as solitary, multiple, well-defined or diffuse opacities in the parenchyma, pleura, hilum or mediastinum. Patients with an abnormal photofluorographical picture, detected by environmental or health controls or upon mass investigation of large groups of the population, usually were lacking in symptoms. For this reason they presented special problems when collecting the specimens due to difficulties in producing sputum.

If the patients' case history, symptoms, signs or X-ray findings gave rise to a suspicion of a malignant disease, then further investigations were undertaken. These included cytological examination of the sputum and various kinds of laboratory investigations, with tomography, bronchography and, if necessary, angiography. Furthermore, bronchoscopy was performed with aspiration of bronchial secretions for cytological examination, followed by biopsy, if possible. In certain cases lung puncture was performed with aspiration and

TABLE 2. *Number of patients with cytological examination of sputum and bronchial secretions by the SH-, AO- and Papanicolaou methods during 1958-1961*

Staining method	Sputum	Bronchial secretions
Proteinbound sulfhydryl (SH) groups	454	121
Acridine-orange (AO) and fluorescence microscopy	88	25
Papanicolaou's method	454	121

microscopical examination of the aspirate. The presence of metastases was shown by biopsy and histopathological examination, for example, gland biopsy according to Daniels (1949) or mediastinoscopy according to Carlens (1960). Sometimes pleural biopsy was carried out with the Vim-Silberman or Abram needle. Finally, various kinds of X-ray examinations were performed in order to locate presumptive metastases of the skeleton or other organs.

If there were reasons to suspect a secondary malignant process of the lungs, pleura or mediastinum then further investigations were undertaken to find out the localization of the primary tumor in other organs through comprehensive clinical, laboratory and X-ray examinations.

As specimens of sputum and bronchial secretions from patients even with a slight suspicion of malignancy were kindly placed at my disposal, the present investigation had the widest possible base for comparing the different staining methods in a large number of pulmonary diseases.

Cytological examination of sputum was performed in 454 patients. Of these, bronchial secretions were also examined in 121 patients. A summary of the 656 final diagnoses in 454 patients with sputum examination and 144 final diagnoses in 121 patients with examination of the bronchial secretions is shown in Table 4. The figures refer to the number of patients with the final diagnosis.

TABLE 3. *Number of specimens of sputum and bronchial secretions examined cytologically by the SH-, AO- and Papanicolaou methods during 1958-1961*

Staining method	Sputum	Bronchial secretions
Proteinbound sulfhydryl (SH) groups	1,073	126
Acridine-orange (AO) and fluorescence microscopy	210	27
Papanicolaou's method	1,073	126

TABLE 4. *Final diagnoses of 454 patients in whom cytological examination of sputum and bronchial secretions was performed*

Diagnosis	Sputum	Bronchial secretions
A. <i>Malignant neoplastic pulmonary diseases</i>		
Primary bronchogenic carcinoma	107	43
Primary pulmonary sarcoma	2	—
Secondary malignant process (pulmonary metastases)	11	4
B. <i>Other malignant diseases without pulmonary metastases</i>	12	3
C. <i>Malignant systemic disease</i>	5	—
D. <i>Benign neoplastic pulmonary diseases</i>	5	2
E. <i>Other diseases of the respiratory tract</i>		
Hemoptysis of uncertain origin	14	4
Rhinitis, sinusitis, laryngitis, bronchitis	47	7
Bronchopneumonia, pleuropneumonia, pulmonary abscess	116	28
Pulmonary tuberculosis (active or healed)	83	14
Pulmonary eosinophilic infiltrates	5	1
Mycosis of the lungs or bronchi	13	5
Bronchiectases, pulmonary emphysema	59	8
Bronchial asthma	33	4
Pulmonary atelectasis, middle lobe syndrom	12	5
Pulmonary cyst	1	—
Spontaneous pneumothorax	4	—
Silicosis	4	—
Sarcoidosis	14	3
Pulmonary fibrosis	1	—
Exsudative or dry pleuritis, pleural empyema, hemothorax, healed pleuritis	30	6
Pulmonary embolism	10	2
F. <i>Diseases of the cardiovascular system</i>	45	2
G. <i>Diseases of the blood</i>		
Venous thrombosis	4	—
Pernicious anemia	5	—
H. <i>Various diseases</i>	11	1
I. <i>Uncertain diagnoses</i>	3	2
Total	656	144

Cytological examination of sputum was performed in a total of 316 men and 138 women between 10 and 88 years of age divided according to sex and age classes as in Fig. 2. Cytological examination of bronchial secretions was performed in 95 men and 26 women between 17 and 76 years of age divided according to sex and age classes as in Fig. 3.

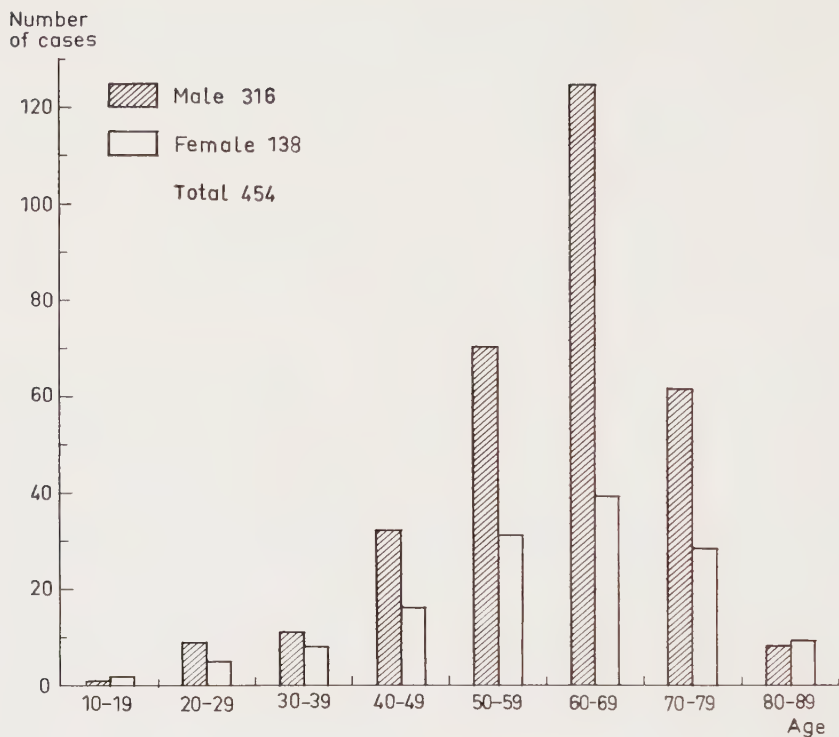


FIG. 2. Distribution according to sex and age of 454 patients, on whom cytological examination of sputum was performed.

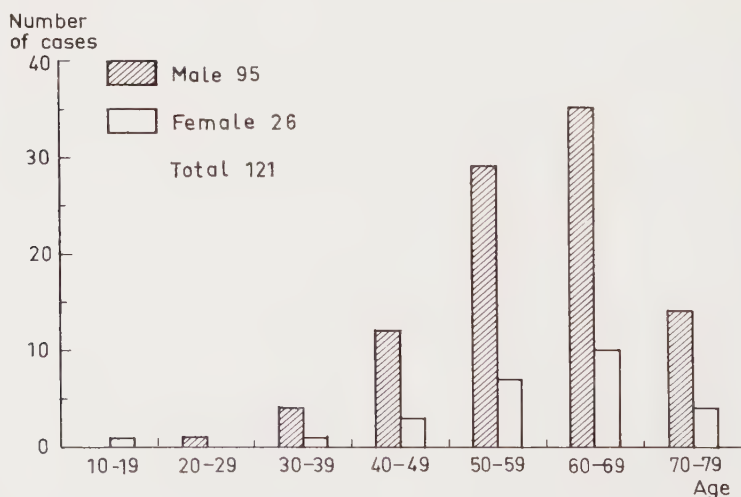


FIG. 3. Distribution according to sex and age of 121 patients, on whom cytological examination of bronchial secretions was performed.



FIG. 4. Distribution according to sex and age of 107 patients with primary bronchogenic carcinoma, on whom cytological examination of sputum was performed.

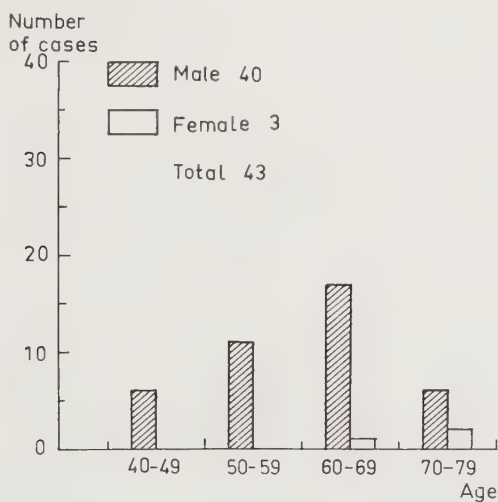


FIG. 5. Distribution according to sex and age of 43 patients with primary bronchogenic carcinoma, on whom cytological examination of bronchial secretions was performed.

TABLE 5. *Tumor localization to the right or the left lung in patients with primary bronchogenic carcinoma and cytological examination of sputum and bronchial secretions*

Localization	No. of patients with cytological examination of	
	Sputum	Bronchial secretions
Right lung	49	22
Left lung	56	21
Both lungs	2	
Total	107	43

In the group A, which comprised all patients with pulmonary malignant diseases, the largest and most important part consisted of primary bronchogenic carcinoma, a total of 107 patients, 90 men and 17 women between 38 and 88 years of age grouped into sex and age classes as shown in Fig. 4. Among these 107 patients cytological examination was also carried out on bronchial secretions aspirated at bronchoscopy in a total of 43 patients, 40 men and 3 women between 42 and 76 years of age grouped into sex and age classes as shown in Fig. 5.

Concerning tumor localization no obvious difference was recognized between the right or the left lung (Table 5). On the other hand, primary bronchogenic carcinoma of the upper lobes predominated over those located in the lower lobes (Table 6). From a histopathological and roentgenological

TABLE 6. *Peripheral and central localization of tumors in 107 patients with primary bronchogenic carcinoma and cytological examination of sputum and bronchial secretions*

Localization	No. of patients with cytological examination of	
	Sputum	Bronchial secretions
Peripheral		
Upper lobes	53	21
Middle lobe	6	4
Lower lobes	28	9
Several lobes	2	—
Central bronchi	18	9
Total	107	43

TABLE 7. *Method used for the first histopathological diagnosis obtained in 91 patients with primary bronchogenic carcinoma and cytological examination of sputum and 38 patients with cytological examination of bronchial secretions*

Method used in obtaining the tissue material for the histopathological examination	No. of patients with cyto- logical examination of	
	Sputum	Bronchial secretions
Bronchoscopy and biopsy from the primary tumor	29	13
Aspiration biopsy from the primary tumor	1	—
Biopsy from metastases	16	6
Operation	20	13
Explorative thoracotomy	4	3
Autopsy	21	3
Total	91	38

point of view the tumor was regarded as originating from the central bronchi in 18 of these patients. In the remainder, the tumor originated from the peripheral parts of the lung.

Histopathological examination was not possible or showed a negative result in 16 patients with cytological examination of sputum and in 5 patients with cytological examination of bronchial secretions. The final diagnosis of these cases was based upon the course of the disease, X-ray and laboratory investigations and other clinical information. Histopathological diagnosis, however, was possible in 91 patients with sputum examination and 38 patients with

TABLE 8. *Results of the initial bronchoscopy performed in 56 patients with primary bronchogenic carcinoma*

Results of bronchoscopy	No. of patients
No tumor visible, no biopsy performed	23
Tumor visible, but biopsy impossible	5
Biopsy performed, histopathological diagnosis negative	14
Biopsy performed, histopathological diagnosis positive	14
Total	56

TABLE 9. *Histopathological type diagnoses in patients with primary bronchogenic carcinoma and cytological examination of sputum and bronchial secretions*

Histopathological type diagnosis	No. of patients with cytological examination of			
	Sputum		Bronchial secretions	
	Men	Women	Men	Women
Squamous cell carcinoma	41	3	21	1
Adenocarcinoma	13	9	2	2
Anaplastic carcinoma (including "oat cell" carcinoma)	22	1	12	—
Alveolar cell carcinoma (bronchiolar carcinoma)	1	1	—	—
Total	77	14	35	3

examination of bronchial secretions, in whom tissue material was obtained at bronchoscopy, biopsy of metastases, operation or autopsy (Table 7).

A total of 56 patients with primary bronchogenic carcinoma were examined bronchoscopically. At the occasion of the initial bronchoscopy 14 of these 56 patients, or 25 %, were diagnosed in this way. In the other cases biopsy could not be performed or the histopathological diagnosis was negative. The results of bronchoscopy are shown in Table 8.

TABLE 10. *Histopathological type diagnosis related to central or peripheral localization in patients with primary bronchogenic carcinoma and cytological examination of sputum and bronchial secretions*

Histopathological type diagnosis	No. of patients with cytological examination of			
	Sputum		Bronchial secretions	
	Central	Peripheral	Central	Peripheral
Squamous cell carcinoma	20	24	9	13
Adenocarcinoma	5	17	1	3
Anaplastic carcinoma (including "oat cell" carcinoma)	8	15	6	6
Alveolar cell carcinoma (bronchiolar carcinoma)	—	2	—	—
Total	33	58	16	22

TABLE II. *The origin of the primary tumor in 11 cases of histopathologically demonstrated pulmonary metastases in patients with cytological examination of sputum or bronchial secretions*

Localization of the primary tumor	No. of patients with cytological examination of	
	Sputum	Bronchial secretions
Breast	1	—
Stomach	1	—
Papilla of Vater	1	—
Pancreas	1	—
Colon or rectum	2	1
Kidney	4	3
Liposarcoma	1	—
Total	11	4

In men, upon histopathological typing of primary bronchogenic carcinoma, there was a preponderance of squamous cell carcinoma and anaplastic (undifferentiated) carcinoma. In women, however, adenocarcinoma was the most frequent histopathological type. Adenocarcinoma and alveolar cell carcinoma (bronchiolar carcinoma) together were more equally distributed between the sexes (Table 9).

With regard to the localization of the tumor the alveolar cell carcinomas (bronchiolar carcinomas) were, as would be expected, peripheral. Among the other carcinoma types the peripheral localization predominated especially in patients with anaplastic (undifferentiated) carcinoma and adenocarcinoma, while the difference in squamous cell carcinoma was of some doubt in this respect. In cases of primary bronchogenic carcinoma originating from the central bronchi the squamous cell carcinomas and anaplastic (undifferentiated) types predominated over adenocarcinomas (Table 10).

The diagnoses of two cases of primary pulmonary sarcomas were first obtained upon operation. The bronchoscopy showed normal endobronchial conditions in these tumors of peripheral localization. Malignant cells, however, were aspirated at lungpuncture in one of these cases.

There were 11 cases with secondary malignant processes of the lungs. All of these were verified upon histopathological examination, either through biopsy or operation of the primary tumor, or through biopsy of metastases or at autopsy. The localization of the primary tumor is indicated in Table 11.

The benign pulmonary tumors, 5 in number, exhibited considerable

diagnostic difficulties, mostly because of their peripheral localization and restricted extent. Only by excluding all other conceivable pulmonary diseases could the existence of a benign or a malignant tumor be determined for the purpose of the final diagnosis, which, in two cases, could first be estimated upon operation.

All other patients with pulmonary diseases had at some occasion been suspected of malignancy, and this was the reason for the cytological examination of the sputum or bronchial secretions.

TECHNIQUE OF COLLECTING SPECIMENS

I. SPUTUM

In the cytological examination of sputum the technique of collecting specimens is of decisive importance. A successful result requires a representative material that originates from the lower parts of the respiratory tract. All specimens containing normal squamous cells only must be excluded for anatomical reasons. These cells are exfoliated from the squamous epithelium located in the anterior part of the nose, the oral cavity, the central and inferior regions of the pharynx and certain parts of the larynx. Neither is the presence of ciliated, cylindrical cells sufficient to consider a sputum specimen representative. It is true that the respiratory epithelium lines the whole bronchial tree and the trachea, but it is also found in parts of the larynx, in the nasopharynx, the largest part of the nasal cavity and in the paranasal sinuses. Clear morphological differences are missing in the respiratory epithelium corresponding to these different localizations, and a sputum specimen containing cells of the respiratory type does not necessarily represent the lower respiratory tract. However, the presence of alveolar cells or sputum macrophages in a sputum specimen is significant and provides a guarantee that the specimen originates from the alveoli and bronchi.

In those patients where symptoms from the respiratory tract were the indication for the cytological sputum examination, the collection of specimens was usually simple to perform. Coughing was an outstanding and constant symptom in these patients, who hardly had any difficulties in producing spontaneously a representative sputum material. Early in the morning, while fasting, the patient was instructed by a physician or a nurse to produce a sputum specimen from the lower respiratory tract through a series of vigorous and efficacious cough blasts. The specimen was collected in a round Petri dish of glass, stainless steel, cardboard or plastic provided with a lid. The addition of a fixative fluid was not necessary when the sputum specimen was processed by the laboratory staff within at least one hour. Specimens which could not be treated immediately, were fixed in 50% ethanol and arrived at the laboratory after 1 to 3 days. Smears from pre-fixed sputum, however, showed several deficiencies and were of inferior quality from a morphological point of view. The preparation was more difficult, and the cells showed considerable

shrinkage and deformity compared with smears from unfixed material. On account of this, trials were performed in a provincial hospital, the samples being collected and the smears prepared in the local hospital laboratory. The smears of fresh material were first fixed for at least 15 minutes in equal parts of ether and 95 % ethanol. Special precautions were taken to prevent the smears from drying and immediately after fixation a drop or two of glycerine was placed over the smear, which was then covered with a clean glass slide and sent by post to the cytological laboratory for staining and screening. The experience of this arrangement was entirely satisfactory.

However, some patients were unable to produce spontaneously sputum with a representative cell material. Sometimes the patients coughed but no expectorate appeared, and sometimes respiratory symptoms were totally lacking. In these patients there was a call for cytological examination of sputum after suspicious or confusing changes in X-ray films or photofluorograms or on account of symptoms from other organs. In this group of patients without expectoration the production of mucus and cough reflexes were stimulated in one or more of the following ways:

1. Slight manual beating dorsally over both lungs during 2-5 minutes; sometimes combined with a body position suitable for drainage of certain bronchi.
2. Peroral or parenteral administration of iodine.
3. Intramuscular injection of guajacol-glycerine-ether (Resyl®, Ciba), 0.1 g (one ampulla à 2 ml).
4. Aerosol inhalation after nebulization of a hypertonic solution of sodium chloride and propylene glycol (NaCl 100 g, propylene glycol 50-200 g, distilled water ad 1000 ml). This method was introduced by Bickerman, Sproul & Barach (1958). According to my own experience, heating of the aerosol is not always necessary. The technique may therefore be modified in such a way that 15 ml of the solution are placed in a glass container (de Vilbiss nebulizer No. 40) coupled to a de Vilbiss air compressor. On a few occasions a special apparatus was used to obtain steam heated to 37°-40°C. With some exceptions no further improvement of the sputum material was obtained compared to that obtained using the aerosol at room temperature. With this method a sufficiently small drop size is obtained to allow passage into the pulmonary alveolar system. The duration of inhalation was varied according to the quantity of the expectorate, usually five to fifteen minutes was sufficient to get an adequate sputum specimen. If difficulties in respiration or other complaints occurred during inhalation, the collection of sputum was immediately interrupted.

2. BRONCHIAL SECRETIONS

From a clinical point of view it is desirable that all patients suspected of having a malignant pulmonary disease are subjected to bronchoscopy. By direct inspection it is possible to obtain detailed information about the tumor size and appearance, localization, extent, relation to surrounding structures, operability, etc. It is essential to make a biopsy, when possible, from the primary tumor, in order to procure an exact histopathological diagnosis and microscopical typing of the tumor which is often of importance for surgical treatment as well as for radio- and chemotherapy. By aspiration of bronchial secretions suitable for cytological diagnosis, the value of the bronchoscopy is increased and the indications for the procedure are widened. This is of special importance in peripherally located processes where no tumor is visible and biopsy is impossible.

In this work all bronchoscopical investigations have been performed by experienced bronchologists in the department of oto-rhino-laryngology of the Uppsala University Hospital. The evening prior to bronchoscopy the patient was premedicated and given orally 25 mg of promethazin.chloride (Lergigan®, Recip) that has a central sedative effect without depression of respiration or disturbance of circulation. One hour before bronchoscopy the patient was given orally 0.25 mg of atropini sulfas in order to diminish the secretion from the glands of the bronchial mucosa and to release the contractions of the smooth muscles of the bronchi. Sometimes 0.5 ml of morphine scopolamine was administered subcutaneously, which also had a sedative, secretion retarding and spasmolytic effect. When half an hour was left, superficial anesthesia of the larynx, trachea and bronchi was commenced using a 1% solution of 2-diethylaminoethyl-4-amino-3-butoxybenzoate hydrochlor. (Novesin®, Wander) which also contains 0.65% sodium chloride. The anesthetic solution was applicated by drip and spray to the larynx with the patient in a sitting position. In some patients bronchoscopy was performed under general anesthesia.

The bronchoscopic investigation was initiated first by inspection of the central bronchi of the healthy lung, then by scrutinizing the bronchial changes of the affected lung. In order to avoid unnecessary admixture of blood in the specimen, the secretions were aspirated prior to a planned biopsy. Sometimes the tumor surface was directly wiped off with a gauze or cotton-wool swab, the collected material was smeared on to glass slides and immediately fixed and stained. At peripherally located processes, without visible changes of the central bronchi, the actual section of the bronchial tree was sometimes washed with Ringer-solution (Salidex®, Pharmacia) and the fluid aspirated in the usual way.

The aspirated secretions were collected either in a glass bowl connected to a sucker, or in a 10 cm long glass tube secured close to the proximal end of the sucker (Pilling & Co., Philadelphia, U.S.A.). The specimen was immediately fixed in 96% ethanol before transport to the cytological laboratory. After centrifuging at 2500 r.p.m. for 10 minutes, a small amount of the sediment was smeared on to albuminized glass slides. The staining of bronchial secretions was performed in the same way as the staining of sputum specimens, according to the methods to be described in the next chapter.

STAINING TECHNIQUES

Unfixed as well as fixed sputum material was scrutinized macroscopically or with a magnifying glass against a dark background in order to separate, if possible, discolored lumps, blood tinged strands or other particles suspected of malignancy (Gloyne, 1935, Wandall, 1944, Hjelt, 1953, Koss, 1961). Suitable quantities of this material were placed between two glass slides and by the application of a gentle pressure were spread out as a thin film on both surfaces. Special instruments designed for making these preparations (Wandall, 1944; Hjelt, 1953), gave an uneven distribution of the material with varying thickness. This made the microscopical work considerably more difficult and did not offer any other advantages. The number of slides, in this work between two and ten from the same specimen, played a subordinate role compared with the number of specimens obtained at different occasions, which was of decisive importance for a satisfactory result (see Chapter VI). Macroscopical scrutinizing of fixed sputum material was made more difficult due to lack of transparency and to obscured blood streaks.

In order to avoid undesirable shrinkage of cells the smears were fixed in a wet state before the material had dried on the slide. This so-called wet-film-technique was introduced by Dudgeon & Wrigley (1935).

I. STAINING FOR THE DEMONSTRATION OF PROTEINBOUND SULFHYDRYL GROUPS IN CYTOLOGICAL SMEARS (THE SH-METHOD)

For the demonstration of proteinbound sulfhydryl(SH) groups of exfoliated cells in sputum and bronchial secretions the author has used the Bahr modification (1957) of the Barnett & Seligman technique (1952) for histochemical work with sectioned material. A survey of the literature concerning different histochemical reactions for SH-groups revealed this method to be the most specific.

The chemical basis and theoretical principles of the SH-staining method is as follows. The freshly prepared wet film smears are fixed in trichloroacetic acid (TCA) and ethanol. Through fixation a strong denaturation of the proteins is obtained and a maximum number of SH-groups are made accessible for reaction with 2,2'-dihydroxy-6,6'-dinaphtyl disulfide (DDD). Fur-

thermore TCA extracts glutathione, cysteine and other substances containing freely reacting SH-groups, prevents oxidizing of proteinbound SH-groups and also cell shrinkage. With DDD in excess at pH 8.5 active SH-groups in fixed cell proteins are oxidized and a colorless substance is formed. This reaction product is insoluble in ether, ethanol and water, while excess DDD and other byproducts can be washed out with organic solvents. The colorless substance now reacts with diazotized 4-amino-3,6-dimethoxy-4-nitroazobenzene (Fast Black Salt K) and *a violet or brown color is produced with a varying intensity directly proportional to the number of accessible SH-groups* (Fig. 1, page 19).

The technical procedure of the SH-staining method is as follows: Before the smears have dried they are immersed in a fixative containing 1 g TCA dissolved in 100 ml of 80% ethanol. After fixation for 24 hours (alternatively quick fixation for 10 minutes) the smears are hydrated in a jar series consisting of xylene, two changes absolute ethanol, 96–80–50% ethanol, remaining at least one minute in each jar. Meanwhile a cover slip is melted to form a container, in which small amounts of staining powder can be weighed. Thus 25 mg of DDD (Schwarz Bioresearch Inc., Mount Vernon, New York, U.S.A. or L. Light & Co. Ltd., Colnbrook, England) is weighed, and the container with DDD is dropped into a 100 ml flask with 15 ml absolute ethanol. The staining powder is easily dissolved, but the solution is somewhat cloudy. Then 35 ml of a 0.1 M-veronal buffer, with pH exactly 8.5, are added and the solution becomes distinctly cloudy. This solution is transferred to a clean cuvette together with the smears and the whole is placed in a thermostat at +5°C. After 1 hour the jar and the smears are allowed to adjust to room temperature for 10 minutes, and the smears are immersed for 5 minutes in each of two jars containing distilled water at a pH of 4.0–4.5, which is achieved by adding a few drops of glacial acetic acid water to the distilled water under control of pH paper (pH must not be below 4.0). The slides are now passed through the alcohol series in ascending concentrations to a jar containing absolutely pure peroxide free ether and then back again through the alcohol series in descending concentration to distilled water (5 minutes in ether, 1 minute in all other solutions). Meanwhile another cover slip is melted and 50 mg of Fast Black Salt K (G.T. Gurr Ltd., London, England) is weighed and dropped into a 100 ml flask with 50 ml of a phosphate buffer at pH 7.0–7.4 (Sörensen). The staining powder is fairly well dissolved during vigorous shaking, and a clear nonfoaming brown solution is obtained and transferred to a clean jar. Before 5 minutes have elapsed since the cover slip container was dropped in the buffer, the smears should be immersed in the staining solution. The staining continues for 2–3 minutes at room temperature and the smears can be seen to take the stain macroscopically. After 10 minutes in running tap water the smears are passed through 95% ethanol to xylene (Fig. 6).

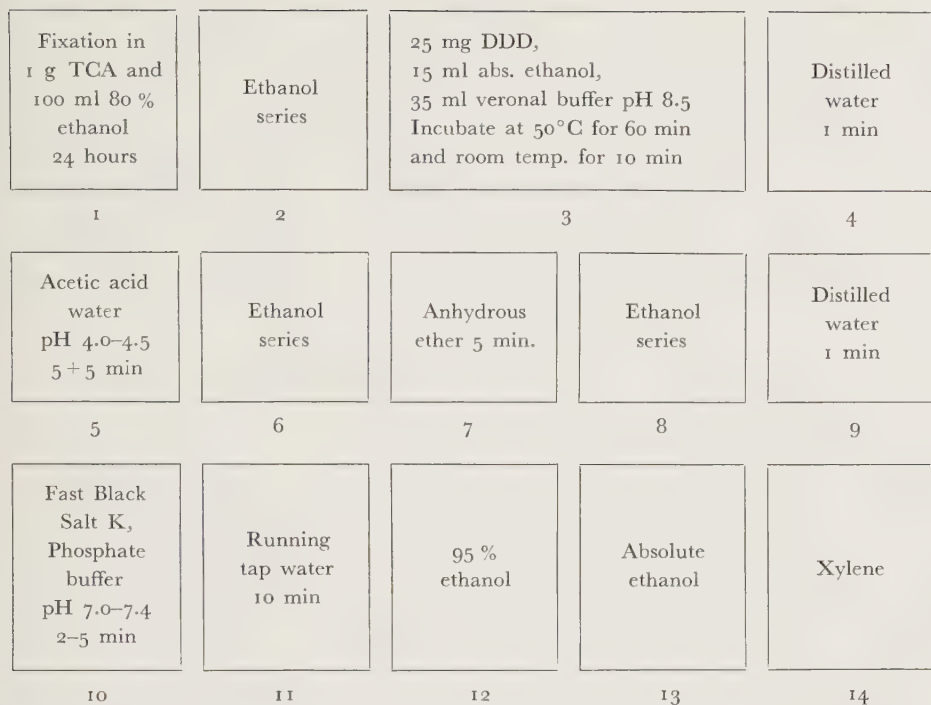


FIG. 6. Staining procedure for the demonstration of proteinbound sulphydryl(SH) groups in exfoliated cells. (Later studies have shown that 10 minutes' fixation time is sufficient.)

Canada balsam was preferred as a mounting medium and provided optimal screening conditions and possibilities for marking of cells with unusual staining patterns or other abnormalities. Heating in a thermostat at +180°C for 2 1/2 minutes followed by immediate cooling in an ordinary refrigerator at +4°C for 5 minutes hardened the Canada balsam and fastened the cover slip firmly and the smears were then ready for immediate cytological screening under the microscope (cf. Manikas & Umiker, 1959). An ideal mounting medium, recently introduced for cytologic use, is Eukitt (O. Kindler, Freiburg i. Br., West Germany) which hardens in 20 minutes without previous heating.

RESTAINING. With the purpose of increasing the examined material, 765 sputum specimens first stained with the Papanicolaou technique were restained by the SH-method. After the cover slips and Canada balsam were removed by xylene, the smears were allowed to remain for 1 or 2 days in a solution consisting of 710 ml 95% ethanol, 280 ml distilled water and 10 ml concentrated hydrochloric acid (HCl), and usually all the stain had disappeared by then. The smears were then treated with trichloroacetic acid (TCA) dis-

solved in ethanol, as mentioned before, and restained by the SH-method described previously. Mounting and screening were performed in the same way as with all other smears. Even after destaining and restaining in this way all cell forms appeared clearly stained in a single color of varying intensity, but with a more brown shade. There was no need to change the principles of cytological assessment, and the results corresponded well with the findings of smears primarily stained by the SH-method. For comparison a special investigation was performed utilizing sputum specimens from every patient suffering from primary bronchogenic carcinoma in order to confirm any possible differences (see Table 15, page 63).

2. STAINING BY ACRIDINE-ORANGE FOR FLUORESCENCE MICROSCOPY (THE AO-METHOD)

After fixation for 15 minutes in equal parts of 95 % ethanol and ether, the smears are hydrated in a series of ethanols, 80-70-50 %, and in distilled water. Then the smears are immersed for 15 seconds in 1 % acetic acid and twice in distilled water. The staining is performed over 3 minutes in a 0.01 % solution of acridine-orange prepared from a stock solution of 0.1 % acridine-orange in distilled water which is diluted with a phosphate buffer of pH 6 ($1/15\text{ M-KH}_2\text{PO}_4 + 1/15\text{ M-Na}_2\text{HPO}_4$). To remove excess of stain the smears are rinsed for 1 minute in the phosphate buffer of pH 6 described above. Eventually the smears are immersed for 1 minute in 0.10 M- CaCl_2 and rinsed in phos-

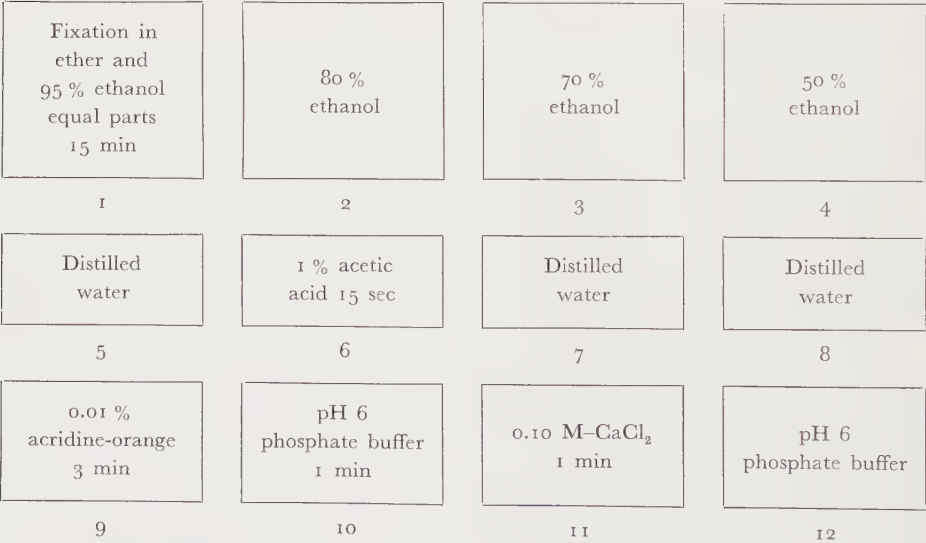


FIG. 7. Acridine-orange(AO) staining procedure for fluorescence microscopy of cytological smears.

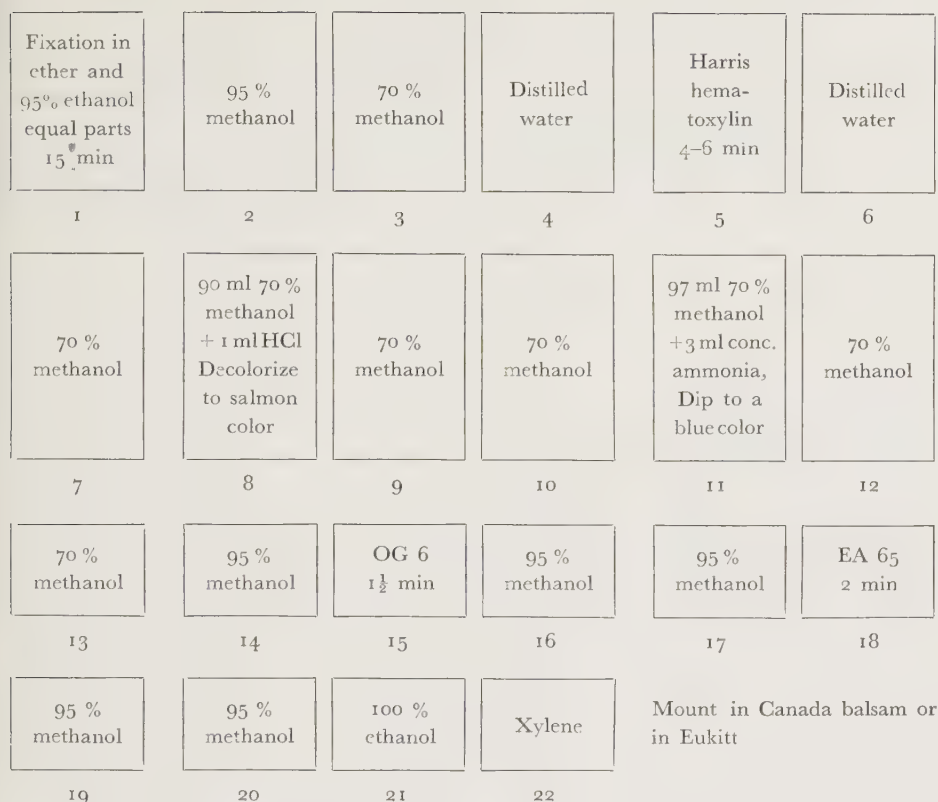


FIG. 8. The Papanicolaou staining procedure for cytological smears.

phate buffer of pH 6, before the microscopical examination is performed (Fig. 7). During screening a cover slip may be placed over the smear if desired. The quality of the stain is of great importance and a British AO-substance (G.T. Gurr Ltd., London, England) was found to be the most suitable in this work. The smears may be stored unmounted and will keep in this state for a couple of months, though with some deterioration of cell morphology. Since fluorescence disappears after some hours another screening demands restaining including fixation, in the ether-alcohol solution. After destaining during 1-2 minutes in 50% ethanol the smears may be restained according to any other morphological or cytochemical method.

As a light source a high pressure mercury lamp (HBO 220, Osram) is used. Between this fluorescence lamp and the object is interposed an exciting filter BG 12, or alternatively BG 3 and BG 38 together, absorbing all wave lengths but the ultraviolet and blue light (usually within the region 300-600 mμ), that passes to the object. Then a metachromatic fluorescence of acridine-

orange is excited. Above the object a suitable barrier filter is used to absorb blue and ultraviolet light which may harm the eye or obscure the fluorescence. The visible fluorescing light, however, passes to the eye and in the microscope the fluorescing object will therefore be seen against a dark background. On account of the weak light intensities in this method all microscopical work has to be done with the eyes fully adapted in a semi-darkened room.

3. STAINING ACCORDING TO THE PAPANICOLAOU TECHNIQUE

In modern exfoliative cytology the Papanicolaou staining technique is the most commonly used method and does not require further presentation here. Since the original description published by Papanicolaou (1942) some modifications have appeared. The method used in this work (Fig. 8) gives a beautiful polychromatic color scale and a good transparency and shows a detailed nuclear morphology and a varying stainability of the cytoplasm. After staining, the smears were mounted in Canada balsam, heated and cooled as described under the SH-method.

THE APPEARANCE OF CELLS IN SPUTUM
AND BRONCHIAL SECRETIONS

1. THE SULFHYDRYL(SH) METHOD

Upon microscopic examination of sputum and secretions obtained at bronchoscopy the cells and other morphological structures present appeared in a single shade of violet or brown after staining with the SH-method. According to previous investigations of tissue material referred to in Chapter I, the different kinds of exfoliated cells in this work acquired a color of varying intensity, thus indicating different concentrations of protein bound SH-groups. An uneven distribution was also observed within each cell.

In patients with *undifferentiated* or *anaplastic bronchial carcinoma* exfoliated cells appeared mostly in clusters composed of highly hyperchromatic forms (Figs. 16 and 26). In the cytoplasm as well as in the cell nucleus and nucleolus the stainability usually exceeded that of the low differentiated types of squamous cell carcinoma and adenocarcinoma. On account of this any cell had an almost homogeneous appearance, and the nuclear chromatin structure was scarcely discernible with low power magnification. However, under high power magnification the nuclear membrane appeared strongly demarcated together with a coarse chromatin network and enlarged and hyperchromatic nucleoli, leaving only a very small zone of cytoplasm. From a morphological point of view this type of cell exhibited the main criteria of malignancy with a high nuclear cytoplasmic ratio, etc. Lymphocytes and cells from the basal layer of the columnar epithelium, which can be very difficult to distinguish from undifferentiated malignant cells with conventional staining methods, stained less intensely with the SH-method than did the malignant cells. This is of great importance in the examination of bronchial secretions, as these usually present special difficulties in this regard due to the presence of numerous cell forms from all layers of the tracheo-bronchial mucosa. The undifferentiated tumor cells can be more easily identified by SH-staining than by conventional morphological staining methods.

Squamous cell carcinomas exfoliated cells with a high SH-activity in the cytoplasm and the nucleus (Figs. 12, 14, 22, 24, 44 and 46). The latter had a fairly constant color intensity and a homogeneous appearance. The cytoplasmic SH-concentration was subject to greater variations, and was sometimes

so high that the nuclear structure was obscured. The nucleolus when present, however, was always of outstanding color intensity and for this reason easy to distinguish. At a lower differentiation with an evident alteration of the nuclear/cytoplasmic ratio in favour of the nucleus and a high frequency of cell clusters, the SH-activity increased in the cytoplasm as well as in the nucleus. On account of their hyperchromasia all these malignant cells were clearly discernible under the microscope with low power magnification.

The grouping, polymorphism, variations of form and size, and the irregularities of the cell nuclei were usually seen at the same time with satisfactory distinctness. There was a good agreement concerning the SH-reaction of tumor cells in smears and tissue sections (Figs. 42, 44 and 46).

Malignant cells desquamated from *adenocarcinomas* within the respiratory tract were characterized by a considerably high stainability with the SH-method (Figs. 18, 28 and 30). The cell nucleus displayed a constant stainability, the large nucleoli especially appearing highly hyperchromatic. The cytoplasm might sometimes seem weakly stained, a fact which may be misleading and probably depends on vacuolization. The vacuolar secretions obviously lacked SH-activity in smeared as well as in sectioned material (Figs. 28 and 48). With lower differentiation the stainability of the adenocarcinoma cells was higher and the concentration of the proteinbound SH-groups was increased. The hyperchromasia of the malignant cells made them easy to identify under the microscope even with low power magnification. The morphology in general was the same as with the conventional staining methods. The SH-activity of exfoliated malignant cells corresponded to that obtained in tumor tissue sections (Figs. 18, 28, 30 and 48).

Large sputum quantities were often characteristic of *alveolar cell carcinoma* or *bronchiolar carcinoma* where the cell flora was dominated by alveolar cells of normal or pathological appearance. The malignant cell nuclei demonstrated a high concentration of proteinbound SH-groups and a strongly stained nuclear membrane was easily discernable together with enlarged and hyperchromatic nucleoli and a more or less prominent chromatin pattern (Fig. 20). The cytoplasm usually displayed a reticulated structure indicating numerous small vacuoles. A similar SH-reaction was demonstrated in the malignant cells of sputum smears and tumor tissue sections (Figs. 20 and 50). The abnormal morphology of solitary cells and cell clusters were easily discernible, as with conventional staining methods.

The typing of the actual cancer form was mostly possible upon the basis of the usual morphologic criteria, which could also easily be recognized by this staining procedure.

Sputum specimens from two cases of primary *pulmonary sarcoma* contained atypical cells with a strong SH-activity in both cytoplasm and nucleus. In

one of these cases there was no doubt about the malignancy of the cells, while in the other case exfoliated cells were only suspect malignant.

Exfoliation from *pulmonary metastases* is not always possible until late in the course of the disease when the tumor growth is penetrating the mucosa. Cytologically malignant cells from the pulmonary metastases in this material were found to be highly hyperchromatic and consequently had a high concentration of proteinbound SH-groups, about the same in the cytoplasm as in the cell nucleus (Fig. 32). Morphologically there was a change in the nuclear/cytoplasmic ratio with enlargement of the malignant cell nucleus, combined with a relatively large cytoplasm usually without vacuolization.

In one case of acute myeloid *leukaemia* with bloodstained sputum numerous solitary cells were recognized with a very strong SH-reaction, probably megaloblasts, to judge from simultaneously performed morphological staining.

Sputum specimens from a patient with *malignant lymphogranulomatosis* contained cells very rich in proteinbound SH-groups which gave rise to a highly suspected malignancy.

Benign pulmonary tumors do not usually exfoliate cells, and the sputum cell flora is normal, as long as the covering bronchial mucosa is intact. If there are, however, possibilities for a desquamation from the benign tumor itself then atypical cells may appear in the sputum. In six cases of benign pulmonary neoplasms SH-stained sputum specimens had a quite normal appearance in two cases, while a slight increase in the concentration of proteinbound SH-groups was noted in two and a more distinct increase in another two cases. For this reason the two last-mentioned were considered to be slightly suspected of malignancy; the same cytological result was obtained by the Papanicolaou staining. One of these patients also had a pernicious anaemia, which might have caused the abnormal cell picture. In the last case the histopathological examination revealed a bronchial adenoma of the carcinoid type. In the SH-stained smears the main features of a benign cell morphology were also to be seen in the exfoliated cells.

Patients with *pernicious anaemia* sometimes had epithelial changes which were recognized in smears with the conventional staining methods as morphologically atypical cells. Further, with the SH-method atypical cells were identified by a slight or moderate increase in the concentration of proteinbound SH-groups on the basis of an increased stainability. A similar condition could be demonstrated in patients with epithelial metaplasia of squamous cell type. A moderately increased SH-reaction in cells exfoliated from these regions gave them an abnormal appearance, but seldom of such a difference as to raise the question of whether malignancy was present or not.

Hitherto the cell forms described usually exhibited a high or moderate in-

crease in the concentration of proteinbound SH-groups when stained by the SH-method. The contrary, however, was found in cells exfoliated from the normal epithelial linings of the respiratory tract.

Cells exfoliated from normal *squamous epithelium* within the respiratory tract showed with SH-staining a rather low SH-concentration without any outstanding observable difference between the cytoplasm and cell nucleus. Basal cells, which are considered to have an increased protein synthesis, had a higher SH-concentration compared to cells from more superficial layers.

Cells from the normal *columnar respiratory epithelium* gave a very weak SH-reaction (Fig. 11), which was particularly true of the goblet cells. As in the case of the squamous epithelium, the basal cells from the columnar epithelium also had a higher staining intensity diminishing towards the ciliated cells of the surface. Usually, the cell nucleus appeared somewhat darker than the cytoplasm. Cubical cell forms from the terminal bronchioles did not generally deviate from the conditions described above.

The *alveolar cells* had a highly varying SH-concentration. These cells have been variously designated in the cytological literature as sputum macrophages, "dust cells", "fat cells", "heart failure cells", histiocytes, etc. The SH-concentration was greatest in the pigment-rich forms, where the cell nucleus might be quite hidden by the deeply stained cytoplasm. This was not unusual in sputum from tumor patients, where the alveolar cells might have phagocytized material from a necrotizing and disintegrating tumor. In spite of the varying stainability these cell forms were very seldom difficult to distinguish from malignant cells because of the cytoplasmic "dust" or pigment particles, vacuolization, sharp and distinct cell boundaries, multinucleation and other morphologically characteristic properties.

Occasionally large multinucleated cells were found in sputum or bronchial secretions. These were interpreted as large *histiocytes* or giant cells of the Langhans type. They exhibited a fairly weak SH-reaction and did not cause any diagnostic problems.

The cell forms of the circulating blood, normal leuco-, lympo-, monocytes and plasma cells appeared very weakly stained and with an indistinct morphology. Erythrocytes also exhibited a low concentration of proteinbound SH-groups but were fully visible and could be recognized without difficulty, which was of a certain diagnostic significance in ulcerating tumors, as erythrocytes act as warning signals in the screening work.

Bacteria of the normal or pathological respiratory tract flora usually exhibited a low SH-activity and tubercle bacilli could not be demonstrated at all in SH-stained smears. A very common finding in sputum from patients with pulmonary diseases were *fungus hyphae* and *blastospores*, mostly of the *Candida* type, which were stained intensely violet or bluish black (Fig. 34);

these could be very easily demonstrated by the staining method for protein-bound SH-groups. Upon the same basis Curschmann spirales were recognized without any difficulty even with low power magnification.

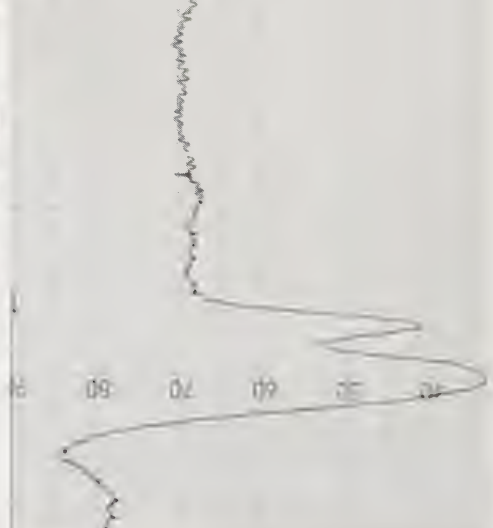
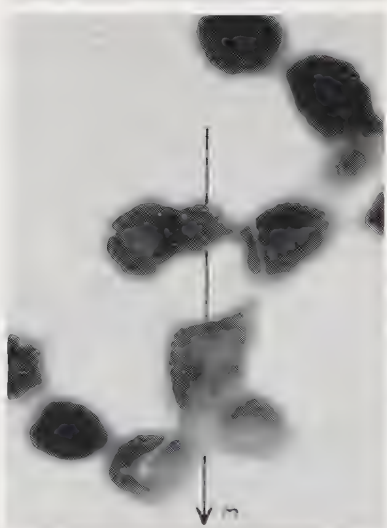
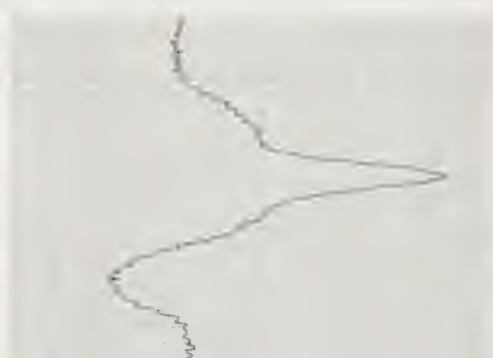
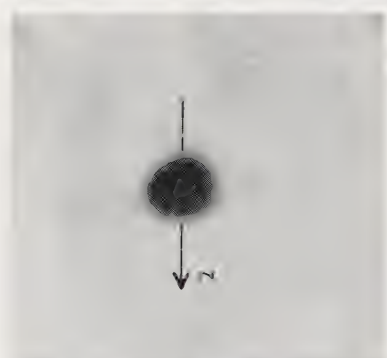
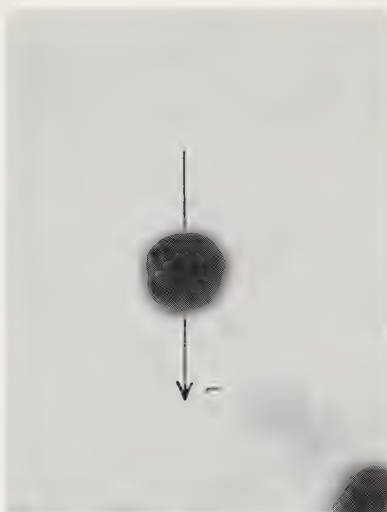
2. MICROPHOTOMETRIC TRANSMISSION MEASUREMENTS OF EXFOLIATED CELLS STAINED BY THE SH-METHOD

Microscopic investigations of smears stained by the SH-method revealed certain differences in the concentration of proteinbound SH-groups in the exfoliated cells originating from various parts of the respiratory tract. Optical estimation of these differences offered hardly any difficulties, even though a degree of uncertainty sometimes arose in particular borderline cases. Further, most patients with primary or secondary malignant diseases of the lungs exfoliated cells with a greatly increased concentration of proteinbound SH-groups (see Chapter VI).

With Bahr's modification of Barnett-Seligman's staining method, in which all cells retain the same color but of varying intensity, it was possible to perform *microphotometric transmission measurements*. Using this method, which has the advantage of being objective, semi-quantitative information was obtained concerning the cellular content of proteinbound SH-groups. Similar measurements were made on histological sections by Moberger & Bahr (1957, 1958), on smear preparations of HeLa cells (malignant cells in tissue culture) by Sandritter & Krygier (1959) and of mouse ascitic tumor cells by Caspersson & Révész (1963).

In this work all transmission measurements were made with a microphotometer for visible light coupled to a Leitz Ortholux microscope using a tungsten filament lamp as the source of illumination (according to Brattgård, 1952). Instead of illuminating the whole field of view an optical system with an inverted objective as a condensor was used (according to Naora, 1955). In this way an illuminated area of $4 \mu^2$ was obtained in the specimen object plane. By moving the object table with the specimen at a constant rate, so that the cells passed through the light beam, the corresponding transmission values were continually registered and reproduced on a recorder (Hallén, 1963; Grampp, Hallén & Rosengren, 1960).

The results of these microphotometric estimations are presented in Figs. 9-11. The minima of the curves indicate a low light transmission and a high concentration of proteinbound SH-groups. Transmission values corresponding to SH-concentrations were obtained for cytologically malignant cells exfoliated from primary bronchogenic carcinoma of the squamous cell type (Fig. 9) and the adenocarcinoma type (Fig. 10), pulmonary metastases of a rectal carci-



noma (Fig. 11) and also for cytologically normal ciliated cells from the bronchial mucous membrane (Fig. 11).

These indirect, objective registrations of the concentrations of protein-bound SH-groups are in complete agreement with the results of normal visual estimation with the light microscope.

3. THE ACRIDINE-ORANGE(AO) METHOD

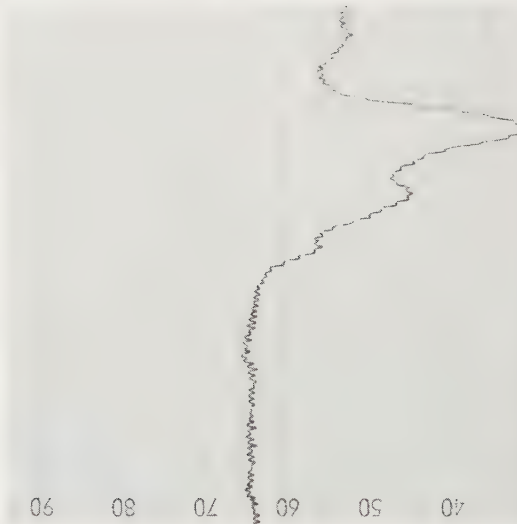
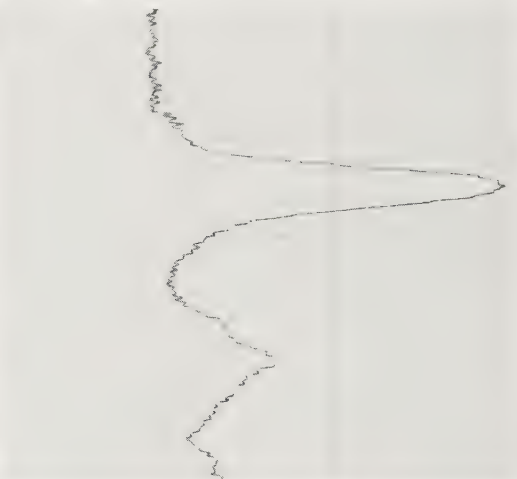
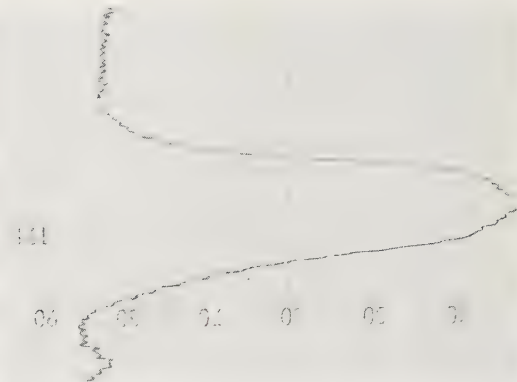
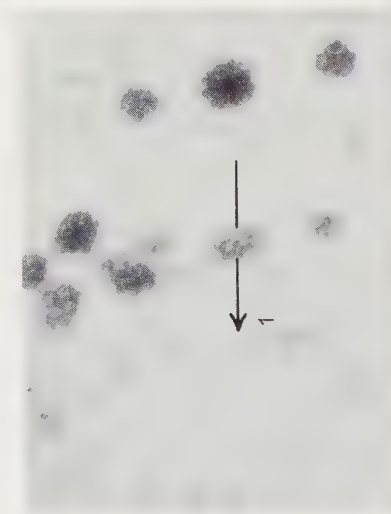
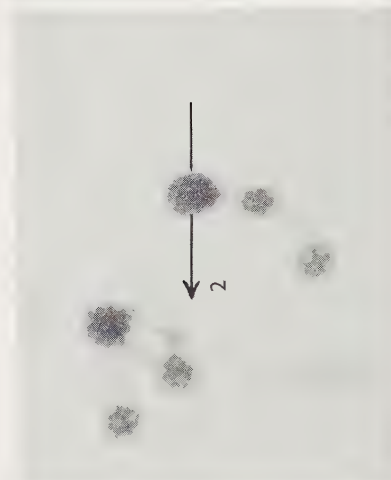
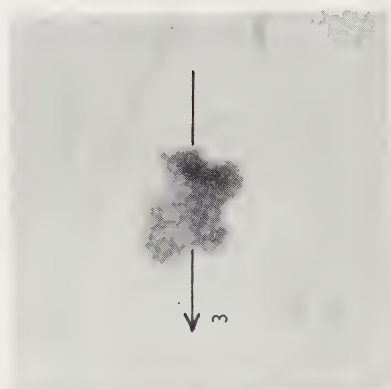
After fluorochromation with acridine-orange cells exfoliated from the respiratory tract produced a red fluorescence in blue or ultraviolet light corresponding to the concentration of ribonucleic acid (RNA) in the cytoplasm and nucleolus. Further, the cell nuclei fluoresced with a yellow color in accordance with the content of desoxyribonucleic acid (DNA). Simultaneously, the morphology of solitary cells and cell clusters was displayed with sufficient distinctness to allow studies of their size and form, as well as changes in the relations between nucleolus, nucleus and cytoplasm.

In cells exfoliated from *undifferentiated or anaplastic carcinomas* a bright red or orange fluorescence of the cytoplasm and nucleolus was produced. Due to the intense fluorescence of the cytoplasm the morphological details of the cell nucleus were hardly recognizable in these small cells of uniform appearance and with only insignificant variations in size. By the usual conventional staining methods these undifferentiated cell forms were sometimes very difficult to distinguish from the normal basal columnar cells of the respiratory epithelium. In the AO-staining the malignant cells exhibited an intensely red fluorescence while the basal cells took on a weak red-brown color, a fact which might contribute to a quick identification of the different cell kinds.

Low differentiated forms of *adenocarcinoma* exfoliated cells with a bright red fluorescence in the same way. With increasing differentiation the color turned more to a carmine red, especially in mucus-producing varieties of adenocarcinoma, where the vacuolized cytoplasm was also supposed to contain acid mucopolysaccharides capable of binding large quantities of the basic stain substance acridine-orange (Figs. 40 and 41).

In *squamous cell carcinoma* a partly new situation arose in the fluorescence of the exfoliated malignant cells. Tumor cells of low differentiation degree produced a fluorescence usually of a yellow-red or orange color which, however, changed to yellow-green with increasing differentiation (Fig. 38). Highly

FIG. 9. Cytologically malignant cells in a sputum smear stained according to the SH-method. Microphotometry was performed on the cells marked 1, 2, 3, and the transmission curves obtained are presented below. A low light transmission value corresponds to a high concentration of proteinbound SH-groups in the cells. (Patient No. 46: squamous cell carcinoma.)



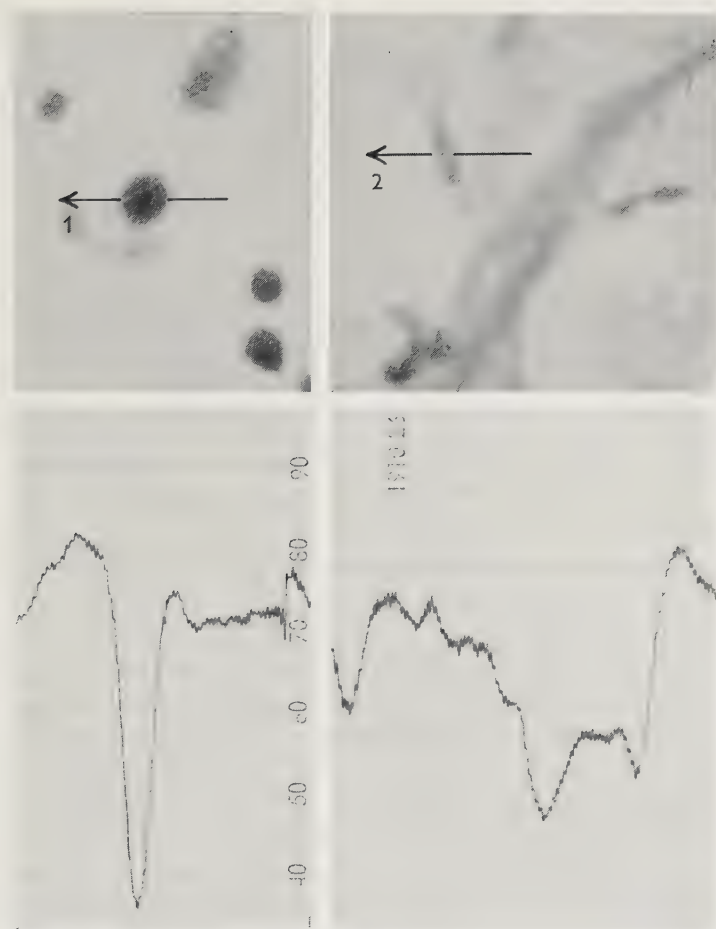


FIG. 11. Cytologically malignant and normal cells in a smear of bronchial secretions stained according to the SH-method. Microphotometry was performed on the malignant cell marked 1 and on the normal ciliated cell marked 2. The transmission curves obtained are presented below. Low light transmission values correspond to a high concentration of proteinbound SH-groups in the cells. (Patient No. 110: rectal adenocarcinoma with pulmonary metastases.)

differentiated types of squamous cell carcinoma exfoliated cells which exhibited only a very faint olive-green fluorescence in the cytoplasm, while the cell nucleus appeared slightly yellow. Thus, these cells had a fairly normal

FIG. 10. Cytologically malignant cells in a sputum smear stained according to the SH-method. Microphotometry was performed on the cells marked 1, 2, 3, and the transmission curves obtained are presented below. A low light transmission corresponds to a high concentration of proteinbound SH-groups in the cells. (Patient No. 69: pulmonary adenocarcinoma.)

appearance apart from the pathological cytomorphology. Ulcerating tumors with necrosis exfoliated numerous degenerated cells with marked nuclear pycnosis, fading nucleus and degenerative changes of the cytoplasm, and were often the object of an advanced keratinization, all signs clearly visible by the Papanicolaou staining technique. None of these characteristic phenomena, however, could be demonstrated with AO-staining and fluorescence microscopy. Furthermore, isolated nuclei without cytoplasm were present in cases of squamous cell carcinoma as well as of adenocarcinoma and anaplastic carcinoma; and, consequently, the material substrate for the characteristic red cytoplasmic fluorescence was quite absent. The polymorphous cell nuclei, however, usually produced a green or yellow-green fluorescence.

Cytologically malignant cells desquamated from *secondary tumors* penetrating the mucosa of the respiratory tract produced a red fluorescence of the cytoplasm and nucleolus in solitary cells and cell clusters (Fig. 41). The exfoliated cells often had a fairly abundant cytoplasm with vacuoles and oval or irregular nuclei with enlarged strongly red fluorescing nucleoli.

Sputum specimens from *benign pulmonary tumors* were examined in only one case of bronchial adenoma of the carcinoid type using the AO-method. There were no deviations from the normal cytology, though with the Papanicolaou technique and SH-method suspected malignant cells were identified.

The normal *squamous epithelium* of the upper respiratory tract exfoliated large, polygonal, superficial cells with abundant faintly green transparent cytoplasm and a central or eccentric, pycnotic or vesicular nucleus with yellow fluorescence (Fig. 36). Cells exfoliated from the basal layers of the squamous epithelium, appeared with a red-brown cytoplasmic fluorescence and large yellow nuclei.

Ciliated cells from both upper and lower respiratory tract exhibited a faint red-brown fluorescence in the cilia. In the goblet cells of the *columnar respiratory epithelium* a reddish fluorescence of the cytoplasm was noted, probably dependent upon the presence of acid mucopolysaccharides. Intermediate and basal cells, solitary or in clusters, had a faint reddish-brown color in the cytoplasm with yellow-green nuclei. The cubical cells from the epithelium of the terminal bronchioles exhibited similar features.

Alveolar cells or sputum macrophages were very polymorphous cells with a green or yellow nuclear fluorescence and a moderate brownish-red color of the cytoplasm, usually containing dust particles or other phagocytized material (Fig. 37).

Among the *cells of the circulating blood* neutrophil and eosinophil granulocytes had the same scarcely detectable green color of the cytoplasm and a yellowish nuclear fluorescence, but were morphologically easy to distinguish from each other (Fig. 37). Lymphocytes, monocytes and plasma cells had an

orange-stained cytoplasm and green nuclei with a normal morphology. Erythrocytes did not fluoresce in ultraviolet light with AO-staining.

Lastly, the most common respiratory tract *bacteria* (not tubercle bacilli) and *fungus hyphae* and *blastospores* from candida species were recognized with great ease due to an intense dominating red fluorescence (Fig. 36).

4. THE PAPANICOLAOU METHOD

The morphology of exfoliated cells stained by Papanicolaou's method is too well known to need further presentation here, existing textbook presentations are quite adequate in this regard. For comparison the microscopic appearance of different kinds of cells with the three staining methods are listed in Table 12 (page 53).

5. CLASSIFICATION OF THE EXFOLIATED CELLS

Upon interpretation of cells detached from their natural environment, no information is, of course, obtainable concerning the structure of the tumor, its infiltration of surrounding tissues, permeation of vessels and its metastases, etc. These are some of the usual criteria supporting the histopathological diagnosis of cancer. It is not always possible to decide if cytologically malignant cells in fact originate from a malignant growth. The highest probability is reached when numerous clusters of cytologically malignant cells are found all over the smear. However, a small number of cells with great abnormalities of the SH-reaction, AO-fluorescence or conventional cell morphology is not always sufficient to provide a positive cytological diagnosis. On the other hand, cells with less pronounced abnormalities may increase the probability of malignancy if they are present in a sufficient number. Thus specimens exhibiting the same cytology may be classified in different ways depending only on a varying number of atypical cells.

In order to attain conformity of the cytological assessment the Papanicolaou classification system was used by the author, and the exfoliated cells were grouped according to the degree of malignancy suspected:

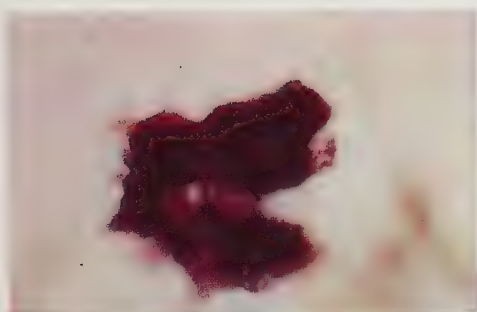
- Class I. Normal cell picture, negative for malignancy.
- Class II. Atypical cytology, but no evidence of malignancy.
- Class III. Cytology suggestive of, but not conclusive for, malignancy.
- Class IV. Cytology strongly suggestive of malignancy.
- Class V. Cytology conclusive for malignancy.

In accordance with previous descriptions of cytology in this chapter this classification system was applied to the SH- and AO-cytology, including the additional cytochemical information obtained from these staining methods. The differences between classes IV and V was slight, in that cell clusters were mostly present in class V but not in class IV. For this reason classes IV and V were considered to be positive from a cytological point of view (Table 12).

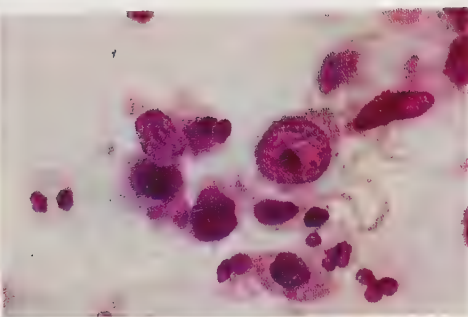
PLATE A
(Sputum smears)

Fig. No.	Cell type	Material	Staining method	Magnification	Pat. No.
12	Squamous cell carcinoma	Sputum	SH	400	54
13	Squamous cell carcinoma	Sputum	Papanicolaou	400	54
14	Squamous cell carcinoma	Sputum	SH (restaining)	400	20
15	Squamous cell carcinoma	Sputum	Papanicolaou	400	20
16	"Oat cell" carcinoma	Sputum	SH	400	27
17	"Oat cell" carcinoma	Sputum	Papanicolaou	400	27
18	Adenocarcinoma	Sputum	SH	201.6	66
19	Adenocarcinoma	Sputum	Papanicolaou	400	66
20	Alveolar cell carcinoma (bronchiolar carcinoma)	Sputum	SH	400	96
21	Alveolar cell carcinoma (bronchiolar carcinoma)	Sputum	Papanicolaou	400	96

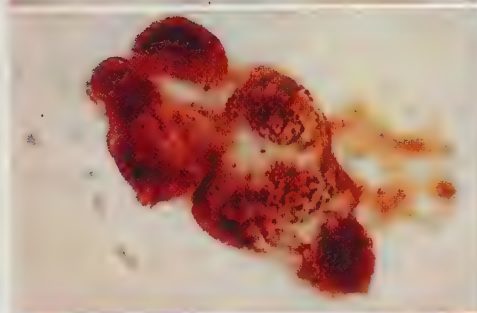
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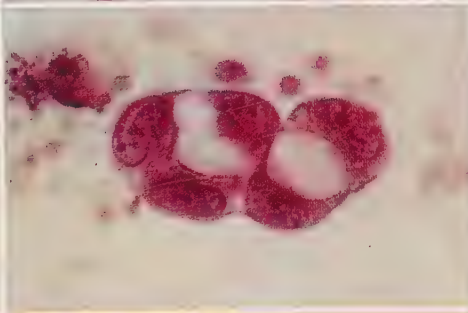
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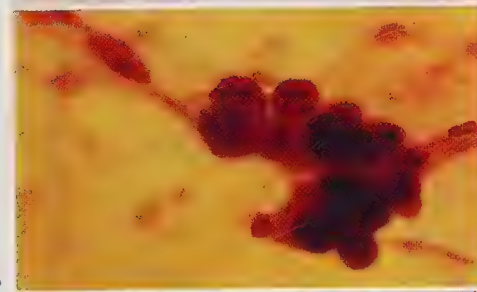
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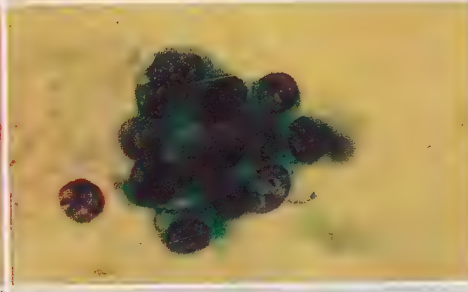
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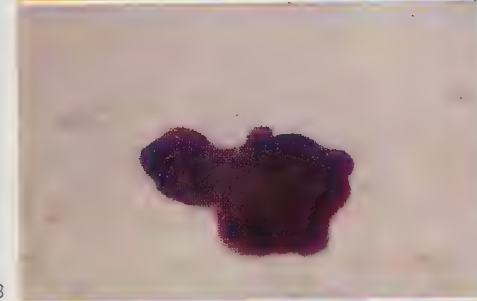
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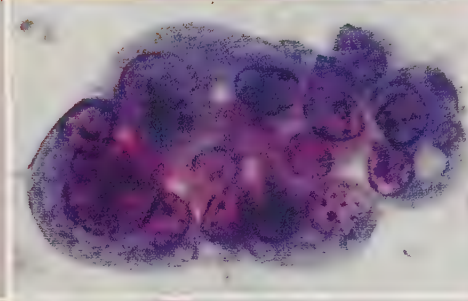
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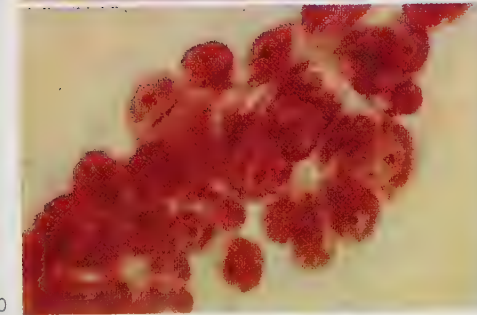
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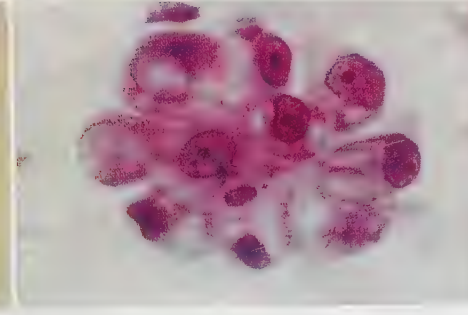
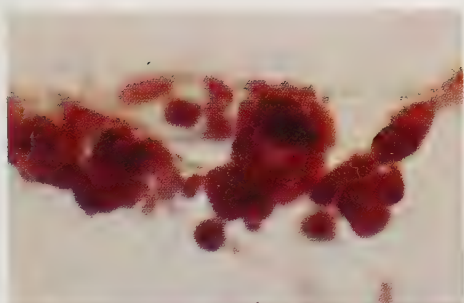
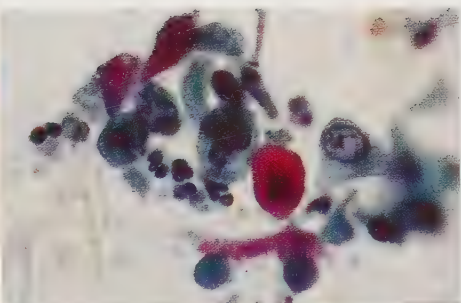


PLATE B
(Smears of bronchial secretions)

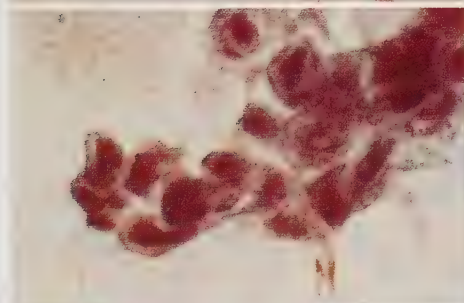
Fig. No.	Cell type	Material	Staining method	Magnifi- cation	Pat. No.
22	Squamous cell carcinoma	Bronchial secretions	SH	400	1
23	Squamous cell carcinoma	Bronchial secretions	Papanicolaou	400	1
24	Squamous cell carcinoma	Bronchial secretions	SH	400	4
25	Squamous cell carcinoma	Bronchial secretions	Papanicolaou	400	4
26	"Oat cell" carcinoma	Bronchial secretions	SH (restaining)	400	36
27	"Oat cell" carcinoma	Bronchial secretions	Papanicolaou	400	36
28	Adenocarcinoma	Bronchial secretions	SH	400	24
29	Adenocarcinoma	Bronchial secretions	Papanicolaou	400	24
30	Adenocarcinoma	Sputum	SH	400	24
31	Adenocarcinoma	Sputum	Papanicolaou	400	24



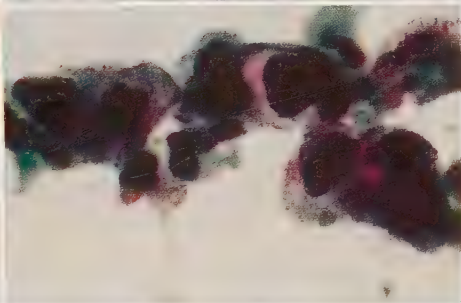
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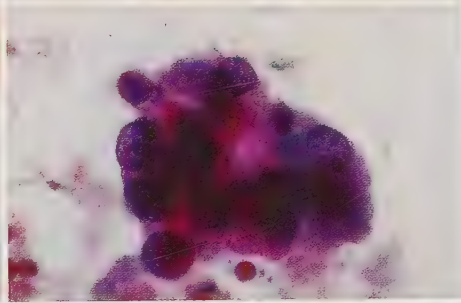
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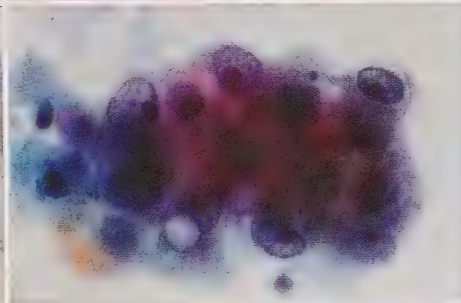
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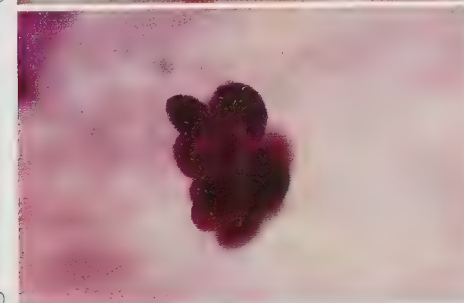
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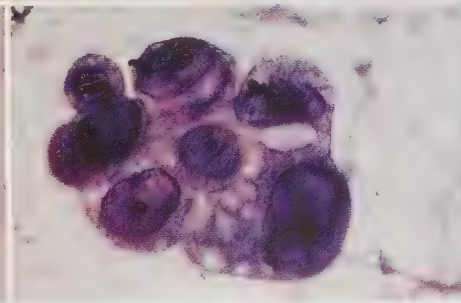
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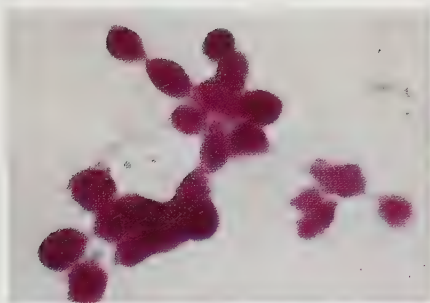


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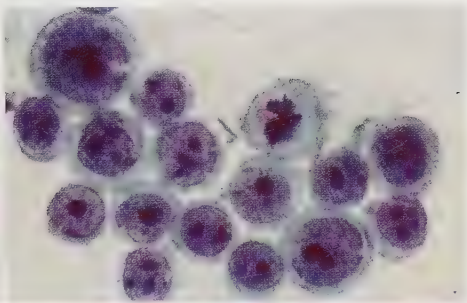
PLATE C
(Sputum and bronchial secretions)

Fig. No.	Cell type	Material	Staining method	Magnification	Pat. No.
32	Rectal adenocarcinoma with pulmonary metastases	Sputum	SH	400	110
33	Rectal adenocarcinoma with pulmonary metastases	Sputum	Papanicolaou	400	110
34	Pulmonary mycosis: fungal hyphae and blastospores of the candida type	Sputum	SH	400	—
35	Adenocarcinoma: fungal hyphae and blastospores of the candida type	Sputum	Papanicolaou	400	74
36	Normal squamous cells and bacteria	Sputum	Acridine-orange	64	—
37	Normal alveolar cells and leucocytes	Sputum	Acridine-orange	64	—
38	Squamous cell carcinoma: malignant cells showing a faint red fluorescence of the cytoplasm	Bronchial secretions	Acridine-orange	102.4	4
39	Adenocarcinoma: malignant cells without a red fluorescence of the cytoplasm	Sputum	Acridine-orange	64	—
40	Adenocarcinoma: malignant cells with a red fluorescence of nucleolus	Sputum	Acridine-orange	102.4	66
41	Carcinoma of the papilla of Vater or alveolar cell carcinoma: malignant cells	Sputum	Acridine-orange	64	114

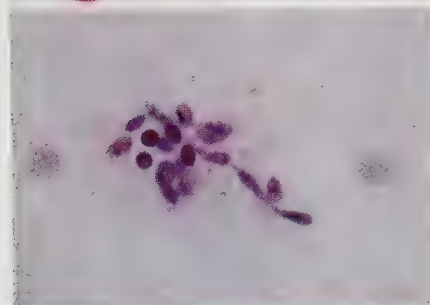
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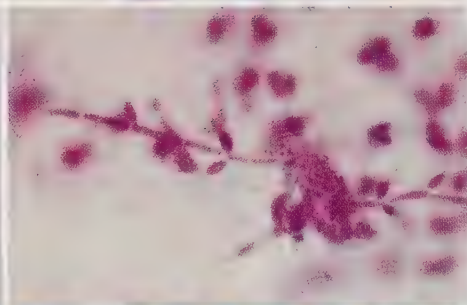
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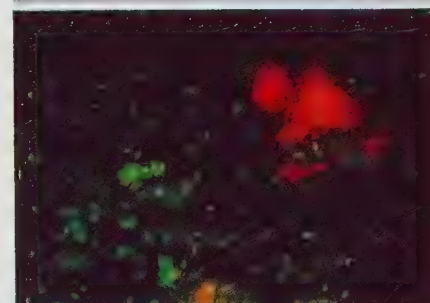
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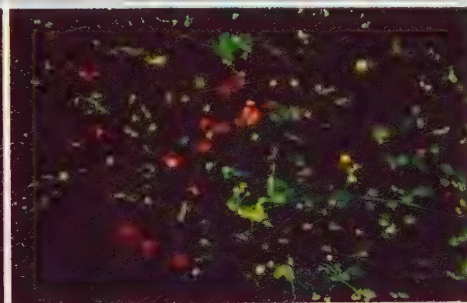
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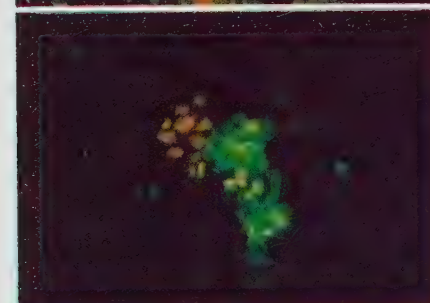
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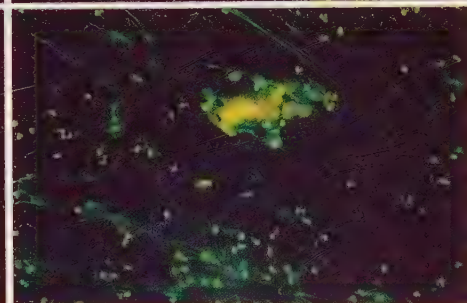
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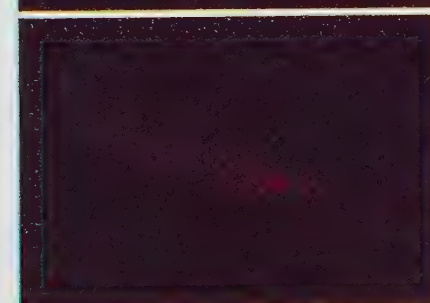
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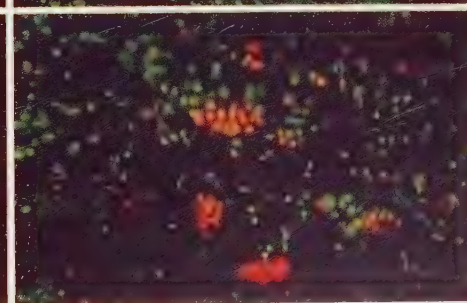
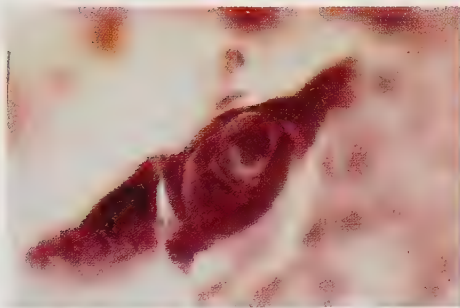
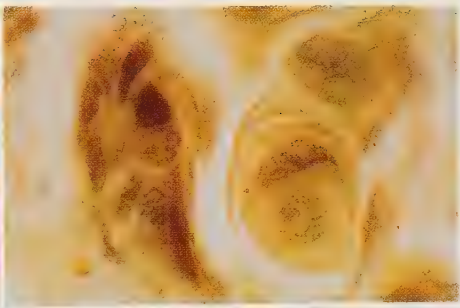


PLATE D
(Tissue sections)

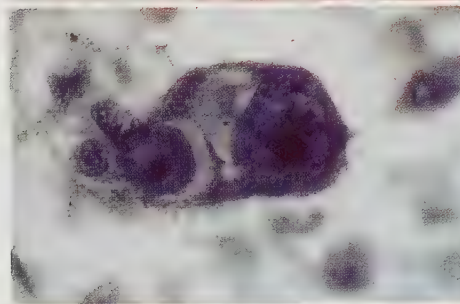
Fig. No.	Cancer type	Material	Staining method	Magnifi- cation	Pat. No.
42	Squamous cell carcinoma	Tumor tissue section	SH	400	62
43	Squamous cell carcinoma	Tumor tissue section	v. Gieson	400	62
44	Squamous cell carcinoma	Smear from the tumor surface	SH	400	62
45	Squamous cell carcinoma	Smear from the tumor surface	May-Grünwald- -Giemsa	400	62
46	Squamous cell carcinoma	Sputum smear	SH	400	62
47	Squamous cell carcinoma	Sputum smear	Papanicolaou	400	62
48	Adenocarcinoma	Tumor tissue section	SH	400	24
49	Adenocarcinoma	Tumor tissue section	v. Gieson	400	24
50	Alveolar cell carcinoma (bronchiolar carcinoma)	Tumor tissue section	SH	400	95
51	Alveolar cell carcinoma (bronchiolar carcinoma)	Tumor tissue section	v. Gieson	102.4	95



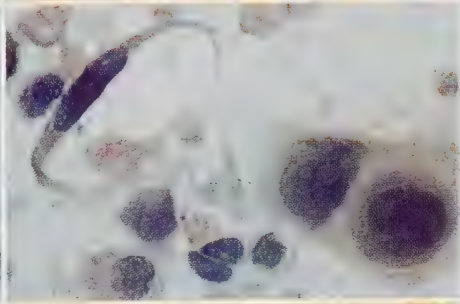
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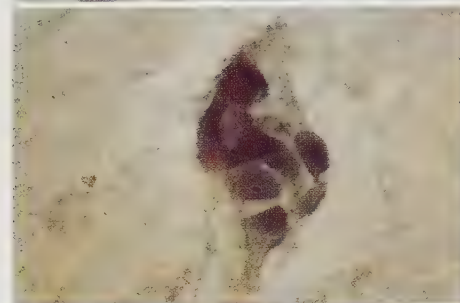
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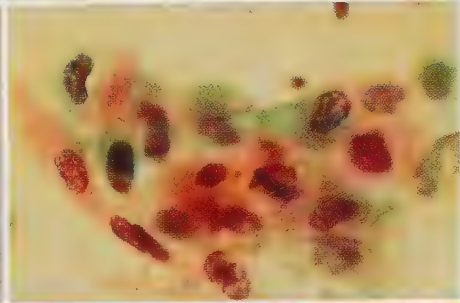
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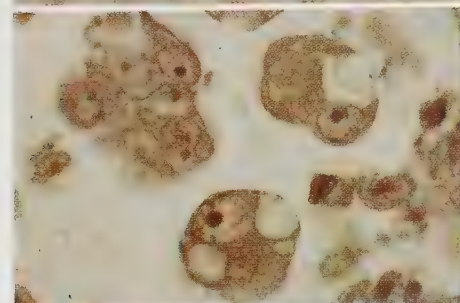
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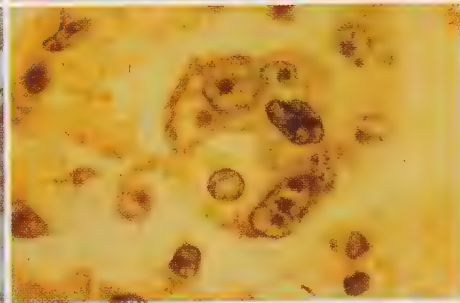
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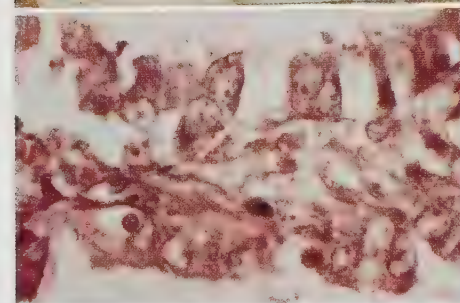
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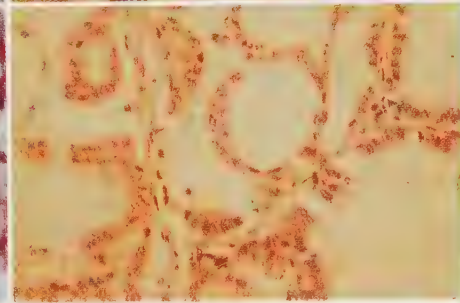
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TABLE 12. *The most common cell types in pulmonary exfoliative cytology according to the SH-reaction, AO-fluorescence and cytomorphology as shown by the Papanicolaou staining procedure*

+ → + + + + +, increasing SH-reaction and concentration of proteinbound SH-groups

Cell type	Classi- fication	SH- reaction	AO-fluorescence	Cytomorphology as shown by the Papanicolaou technique
Squamous cell carcinoma	IV-V			Polymorphous cells
Nucleolus		- + + + +	Orange	Solitary, multiple, black
Nucleus		+ + + + +	Yellow-green	Polymorphous, black, bluish black
Cytoplasm		+ + + + +	Olive-green, orange	Abundant, orangeophil
Adenocarcinoma	IV-V			Polymorphous cells
Nucleolus		+ - + + +	Intensely red	Solitary, multiple, black
Nucleus		+ + + + +	Yellow	Round, polymorphous, bluish black
Cytoplasm		+ + + + +	Carmine-red	Abundant, vacuolized, basophil
Bronchiolar carcinoma	IV-V			Round, irregular cell forms
Nucleolus		+ + + + +	—	Solitary, multiple, bluish black
Nucleus		+ + + + +	—	Round, irregular, blue
Cytoplasm		+ + + + +	—	Abundant, reticulated, basophil
Anaplastic carcinoma	IV-V			Round, oval cells of varying size
Nucleolus		+ + + + +	Intensely red	Solitary, multiple, black
Nucleus		+ + + + +	Yellow	Round, oval, black
Cytoplasm		+ + + + +	Bright red	Scanty, orangeophil, basophil
Squamous cell metaplasia	II			Oval or polygonal cells
Nucleus		+ +	Yellow	Dyskaryotic
Cytoplasm		+ +	Reddish-brown	Eosinophil, (carmine) red
Squamous epithelium (superficial)	I			Large, polygonal cells
Nucleus		+	Green or yellow	Round, vesicular, blue
Cytoplasm		+	Faint green	Abundant, eosinophil
Squamous epithelium (basal)	I			Round, oval cells of moderate size
Nucleus		+ +	Yellowish-green	Round, blue
Cytoplasm		+ +	Brownish-yellow	Fairly abundant, basophil
Columnar epithelium (superficial)	I			Elongated, slender, ciliated cells
Nucleus		+	Yellowish-green	Round, oval, blue
Cytoplasm		+	Reddish-brown	Sparse, basophil (eosinophil)
Goblet cells	I			Elongated, stout cells
Nucleus		+	Yellow	Round, triangular, blue
Cytoplasm		(+)	Brownish-red	Vacuolized, basophil
Columnar epithelium (basal)	I			Small, round or angular cells
Nucleus		+ +	Yellowish-green	Round, blue
Cytoplasm		+ +	Reddish-brown	Scanty, basophil
Alveolar cells	I			Polymorphous cells
Nucleus		+	Green or yellow	Round or irregular
Cytoplasm		varying	Reddish-brown	Abundant, reticulated, eosino- or basophil

Table 12 (continued)

Cell type	Classi- fication	SH- reaction	AO-fluorescence	Cytomorphology as shown by the Papanicolaou technique
Neutrophil granulocytes	—			Polymorphous
Nucleus		+	Yellow	Segmented, polymorphous
Cytoplasm		(+)	Unstained	Faintly neutrophil
Eosinophil granulocytes	—			Polymorphous
Nucleus		+	Yellow	Segmented
Cytoplasm		(+)	Reddish-brown	Eosinophil, granulated
Lymphocytes	—			Round, oval, monomorphous
Nucleus		+	Yellowish-green	Round, blue
Cytoplasm		+	Faintly red-brown	Scanty, basophil
Plasma cells	—			Oval
Nucleus		+	Green	Round, oval, eccentric
Cytoplasm		+	Reddish-brown	Abundant, basophil
Erythrocytes	—	+	No fluorescence	Round, light red
Bacteria	—	+	Red	Blue
Fungal hyphae and blastospores		++++	Red	Blue

SH-REACTION. Differences in the SH-reaction, and consequently also in the concentration of the proteinbound SH-groups, were graded in an increasing scale from + to + + + + + (Table 12). The color intensity corresponded in this respect to the light transmission curves obtained upon microphotometry related in Chapter V: 2, Figs. 9–11. Thus, the + SH-reaction means a low concentration of proteinbound SH-groups, as encountered in the normal ciliated cells illustrated in Fig. 11. One of these cells, marked 2, was selected for microphotometry and the results obtained showed a high light transmission concluding a low SH-concentration. A + + + SH-reaction is presented in Fig. 10, where the cell marked 1 was subjected to microphotometry. The corresponding transmission curve showed a moderately increased SH-concentration. Finally the results of microphotometry of the other marked cells in Figs. 9–11 indicated a low light transmission corresponding to a + + + + + SH-reaction and an elevated protein sulfhydryl level. Besides this staining reaction the gross morphology was also used for the classification.

RESULTS OF CYTOLOGICAL ASSESSMENT

I. THE SULFHYDRYL(SH) METHOD

A. Sputum

Cytological investigation of sputum after staining by the SH-method was undertaken on 1,073 specimens with a representative material from 454 patients (Tables 2 and 3, page 22), i.e. an average of 2.5 specimens per patient. The comprehension and the composition of the material is presented in Chapter II.

378 specimens containing a representative material from 107 patients with *primary bronchogenic carcinoma* were examined by the SH-method, that is, approximately 3.5 specimens from each patient. In Table 13 a (page 56) and Fig. 52 (page 58) the results after examination by the SH-technique have been compiled with results achieved by the AO- and Papanicolaou methods. It was soon evident that one sputum specimen only was seldom sufficient in achieving a final diagnosis, especially if many specimens consisted only of squamous epithelial cells of the pharynx and oral cavity, and thus lacked cell material that was representative of the lower respiratory tract. *The table and the figure demonstrate the importance of frequent sputum collection, in so far as the number of positive reports could be considerably raised if representative sputum specimens were collected on several different occasions.* More than three specimens, however, usually gave no significant improvement, even if a new series of specimens were necessary in doubtful cases.

Among the three different staining methods which were used the SH-method afforded the best results; this was due to the fact that the malignant cells could be demonstrated in up to 83% of the cancer cases if five specimens were examined, and to 81% if three specimens were examined.

Class I. All of the 107 patients with primary bronchogenic carcinoma had, on some occasion, atypical cells with an increased concentration of protein-bound SH-groups, excepting five cases of pulmonary adenocarcinoma with a peripheral localization (Nos. 67, 68, 70, 72, 79) and one case of anaplastic bronchogenic carcinoma (No. 47), constituting 5.6% of the cancer patients. With none of the three staining methods used could cytologically malignant cells be recognized in representative specimens from cases Nos. 47, 67, 68, 79.

TABLE 13a. *Distribution of 107 patients with primary bronchogenic carcinoma with respect to the results of cytological examination of sputum using the SH-, AO- and Papanicolaou staining methods*

Method	Classi- fication	No. of patients with highest result classification in					
		First specimen	First to second	First to third	First to fourth	First to fifth	All specimens
SH-method	I	17	5	1	—	—	6
	II	6	7	2	2	1	4
	III	26	10	6	5	3	16
	IV	20	17	11	10	6	16
	V	38	35	28	17	13	65
	Total	107	74	48	34	23	107
Positive specimens (IV and V) in per cent		54	70	81	79	83	76
AO-method	I	14	5	1	1	1	10
	II	3	3	2	1	1	3
	III	—	2	1	—	—	3
	IV	3	2	1	1	—	3
	V	3	4	3	1	1	4
	Total	23	16	8	4	3	23
Positive specimens (IV and V) in per cent		26	38	50	50	33	30
Papanicolaou method	I	31	11	5	3	2	12
	II	10	10	8	7	6	9
	III	19	8	4	5	6	7
	IV	14	9	8	3	—	17
	V	33	36	23	16	9	62
	Total	107	74	48	34	23	107
Positive specimens (IV and V) in per cent		44	61	65	56	39	74

Classification

Class I. Normal cell picture, negative for malignancy.

Class II. Atypical cytology, no evidence of malignancy.

Class III. Cytology suggestive of, but not conclusive for, malignancy.

Class IV. Cytology strongly suggestive of malignancy.

Class V. Cytology conclusive for malignancy.

None of these patients had more than two representative specimens, which usually are insufficient in pulmonary cytodagnosis.

Class II. In another four cases (3.7 %) there were no possibilities of finding cytologically malignant or suspected malignant cells using the SH-method (Nos. 9, 40, 65, 107). However, in sputum atypical cells were recognized with

TABLE 13*b*. Differences in per cent numbers obtained by comparing the results of the three cytological staining methods given in Table 13*a*

Methods	Difference of per cent number in					All specimens
	First specimen	First to second	First to third	First to fourth	First to fifth	
SH-AO-method	28*	32*	31	29	50	46***
SH-Papanicolaou method	10	9	16	23	44**	2
Papanicolaou-AO method	18	23	15	6	6	44***

* $0.01 < p < 0.05$ ** $0.001 < p < 0.01$ *** $p < 0.001$.
(Statistical testing according to the χ^2 -method with one degree of freedom.)

a concentration of proteinbound SH-groups too low to provide a suspicion of malignancy. For various reasons the number of specimens examined was also low in these patients. A more frequent sputum collection would probably have increased the value of this diagnostic procedure.

Class III. By the SH-method cells suspected of malignancy were recognized, showing a moderate concentration of proteinbound SH-groups in 16 of the cancer cases (15.9%): seven were squamous cell carcinomas, three adenocarcinomas, five were of the anaplastic type, and histopathologic diagnosis was unobtainable in one case.

Classes IV and V. Gravely atypical cells of a probable or conclusive tumor character were found in the remaining 81 of the patients (76%) suffering from primary bronchogenic carcinoma.

In one patient with *primary pulmonary sarcoma* (No. 108) atypical cells were found in sputum with an SH-reaction conclusive for malignancy. In one case of rhabdomyosarcoma (No. 109), probably primary of the lung, sputum specimens contained cells of a moderate SH-content with a suspicion of malignancy.

Of 23 patients with *extrapulmonary carcinoma or sarcoma* 11 had metastases in one or both lungs (table 4 and 11). With the cytological sputum examination equivalent results regarding malignancy were obtained by the SH-method and the Papanicolaou technique. In two patients with hypernephroma (No. 120) respectively carcinoma of the colon and rectum (No. 117), the sputum specimens had only a normal cell picture upon SH-staining. In one case of liposarcoma with a large metastasis of the lung (No. 119) atypical cells were recognized in sputum without the increased SH-reaction giving suspicion of malignancy. A moderately increased SH-reaction in slightly

Positive report
%

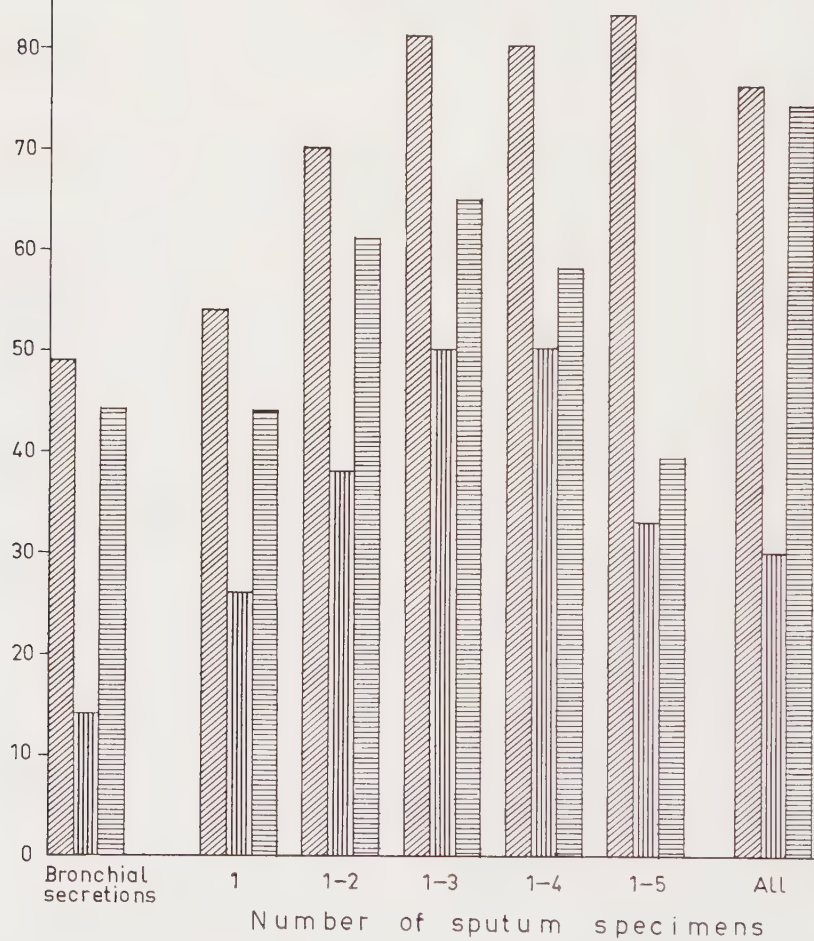
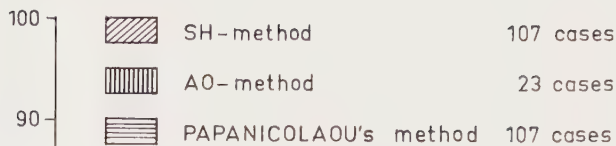


FIG. 52. Results of cytological examination of sputum and bronchial secretions in primary bronchogenic carcinoma using the SH-, AO- and Papanicolaou staining methods.

atypical cells gave rise to suspicion of malignancy in one case of gastric ulcer with malignant changes, adenocarcinomatous in type, which had spread to the pleura and lungs (No. 116). In the remaining seven patients with pulmonary metastases (Nos. 110, 111, 112, 113, 114, 115, 118) a very high concentration of proteinbound SH-groups was found in the cells exfoliated into sputum. At the same time, morphological abnormalities of various kinds were found constituting a support of the cytodiagnosis of malignancy.

Of the remaining 12 patients with an extrapulmonary carcinoma or sarcoma, where no pulmonary metastases could be found at the moment of the investigation, ten had a normal sputum cell picture. The SH-reaction of the exfoliated cells showed small variations but was always very low, and there were no signs of malignancy in the cells. Of special interest were two patients in whom cytologically malignant cells were found in sputum by both SH- and Papanicolaou staining methods. One of these patients had a carcinoma of the thyroid with a metastasis in the nasal cavity, which was first recognized at the cytological sputum examination (cf. Stahle & Wiman, 1962). The second patient had carcinoma of the oesophagus with perforation to the trachea.

In one case of acute myeloid *leukemia* with great hemoptyses and leukemic infiltrations in both lungs (No. 121) numerous solitary cells with very strong SH-activity were identified in SH-stained sputum smears. To judge by the Papanicolaou staining, these strongly hyperchromatic cells were considered to be myeloblasts. Sputum specimens from another patient with a chronic lymphatic leukemia (No. 122) showed quite normal cells upon SH-staining. One patient with *malignant lymphogranulomatosis* and hemoptyses (No. 123) had in one sputum specimen numerous solitary cells with a high concentration of proteinbound SH-groups which were interpreted as being highly suspicious of malignancy. In two patients, one with *myelomatosis* (No. 124), and the other with *mycosis fungoides* (No. 125), no SH-positive cells could be found in the sputum.

Of five patients with *benign pulmonary tumors*, two had quite normal cell pictures in cytologically examined sputum specimens. One of these two patients (No. 126) had a hamartoma verified at operation. The other patient (No. 129) had a clinical diagnosis of bronchial adenoma or probably tuberculoma. One patient (No. 128) with an atypical cell picture without suspicion of malignancy had a rounded shadow in the right lung unchanged in the X-ray film over many years. Histopathological diagnosis was lacking, but clinical investigations and the course of the disease corresponded to a benign pulmonary tumor.

Another patient had a pernicious anemia (No. 130) and a clinically benign pulmonary tumor without a histopathological diagnosis. However, in one

sputum specimen cells were recognized with a slightly raised SH-reaction and an abnormal morphology of a type sometimes seen in pernicious anemia with epithelial changes.

Of special interest was a patient with a benign pulmonary tumor (No. 127) where the cytological sputum examination with the SH-method showed atypical cells with so great a concentration of the proteinbound SH-groups that malignancy had to be suspected. Segmental resection was performed on account of a suspected bronchogenic carcinoma. The histopathological examination, however, revealed a carcinoid of the lung with a penetration of the capsula and a certain degree of nuclear polymorphism.

In *other diseases of the respiratory tract* (312 patients) the sputum cell picture was usually normal with the SH-method. In 47 cases, however, clear abnormalities were recognized and cells with an increased concentration of proteinbound SH-groups were identified. Since possibilities exist in this situation for erroneous interpretation regarding a diagnosis of malignancy a special analysis of these conditions was necessary.

Class V. In four patients strong SH-positive cells and cell clusters in the sputum were interpreted as probably malignant, although no malignant tumor could be diagnosed using other methods of investigation and the clinical course was not conclusive for cancer. One of these patients was a 65-year-old man with a double-sided, possibly healed, pulmonary tuberculosis and a great number of homogenous, well defined, rounded lesions, of a histopathologically unclear nature, probably chronic, necrotic bronchopneumonias. One patient was a 53-year-old man with a large right-sided lobar pneumonia, who was treated with antibiotics with a successful outcome, the X-ray opacity disappearing. One patient was a 70-year-old man who was treated in the hospital for hypertension and cardiosclerosis. He died of pulmonary embolism. A 60-year-old man, with a broncho-pneumonia, recovered after treatment with antibiotic drugs.

Class IV. Another four patients upon cytological examination with the SH-method had a cell picture with a very strong suspicion of malignancy, although clinically no malignant tumor was diagnosed. The first was a 51-year-old man with a right-sided exsudative pleuritis which disappeared after treatment with specific chemotherapy. The second was a 57-year-old man who had a left-sided bronchopneumonia with atelectases and a prolonged course. The third was a 52-year-old woman with a serious bronchial asthma which was observed over many years, but there were no clinical or other signs of cancer. The last patient was a 73-year-old man who had a double-sided healed pulmonary tuberculosis and a bronchopneumonia, which disappeared upon un-specific chemotherapy.

With the SH-method sputum smears from these eight patients were errone-

TABLE 14. 68 clinical diagnoses in 39 patients without a malignant disease, but with cells suspected of malignancy in the sputum. The SH-method

Bronchopneumonia, pleuropneumonia, pulmonary abscess	19
Pulmonary tuberculosis	9
Allergic conditions	8
Bronchitis, acute or chronic	4
Sarcoidosis	2
Bronchiectases	3
Pulmonary emphysema	2
Pulmonary atelectasis	3
Spontaneous pneumothorax	2
Exsudative pleuritis, pleural empyema, healed pleuritis	5
Cardiovascular diseases	4
Hemoptysis	3
Pernicious anemia	1
Various diseases and unclear diagnoses	3
Total	68

ously interpreted as cytologically positive. Related to the total number (454) of the patients examined with a representative sputum material these *false positive cancer diagnoses* totalled 1.7 %.

Class III. A cytological report of a suspected malignancy on account of a moderately increased concentration of proteinbound SH-groups associated with certain morphological abnormalities was registered in 39 patients (8.5 %) without other signs of a malignant tumor. In these patients 68 clinical diagnoses were distributed as shown in Table 14.

CONCLUSIONS

Upon the examination of sputum by the SH-method cytologically malignant cells were demonstrated in optimally 83 %, and suspected malignant cells in 16 % of 107 cases with primary bronchogenic carcinoma. False positive cancer diagnoses were reported in 1.7 % of all the 454 patients examined.

B. Results of cytological examination after restaining of sputum smears by the SH-method

In this work 308 sputum specimens from 133 patients were primary stained by the method for the demonstration of proteinbound SH-groups. Through destaining in hydrochloric acidic ethanol according to the description on page 37, a further 765 Papanicolaou stained specimens from 321 patients were available for staining by the SH-method. In primary SH-stained sputum smears the exfoliated cells adopted a single violet or brown color. In the sputum smears which were restained by the SH-method the cells appeared

in a violet-brown or brown color of varying intensity and with unchanged morphology. There were reasons to presume that principally the same course of chemical reactions took place at the restaining as with the primary staining by the SH-method, and that the distribution of proteinbound SH-groups were the same after both procedures.

As a support for this opinion a separate study was performed of all sputum specimens from the 107 patients with primary bronchogenic carcinoma. Primary and secondary staining by the SH-method were considered as quite different staining methods. The results were compiled and assessed separately, using the same cytological classification. In this respect consideration was taken of the color intensity of the cells as a measure of the concentration of proteinbound SH-groups and to the morphology of the cells in their normal or abnormal appearance. The result of this study is shown in Table 15. In 42 patients the sputum specimens were primary stained by the SH-method, while restaining by the SH-method was performed on specimens from a total of 93 patients. A fairly good agreement was obtained at the cytological assessment of smears treated with these two different procedures.

Equally good agreement was obtained in non-malignant diseases. Among the eight patients with false positive cancer diagnoses mentioned on page 60, three erroneously interpreted sputum smears were primary SH-stained, while five were restained.

CONCLUSIONS

Thus, there are reasons to accept primary and secondary SH-stained smears as being equivalent for cytological cancer diagnosis.

C. *Bronchial secretions*

Cytological examination of secretions aspirated at bronchoscopy was performed in 126 specimens from 121 patients using SH-staining. Specimens with poorly treated or insufficient material were excluded, as well as specimens where possibilities for comparison between the different staining methods were lacking. In a few cases secretions from more than one occasion of bronchoscopy were examined resulting in different cytological interpretations. In the same patient the highest classification was used for the compilation of the results. No definite conclusions could be drawn in regard to repeated specimen collection due to the small number of specimens available. The comprehension and the composition of the material is presented in Chapter II.

Bronchial secretions from 43 patients with *primary bronchogenic carcinoma* were examined by the SH-method. In Table 16 (page 64) and Fig. 52 (page 58) a compilation is presented of the results of staining by all three methods.

TABLE 15. *Distribution of 107 patients with primary bronchogenic carcinoma with respect to the results of cytological examination using primary and secondary SH-staining of sputum smears*

Method	Classi- fication	No. of patients with highest result classification in					All specimens
		First specimen	First to second	First to third	First to fourth	First to fifth	
Primary staining by the SH-method	I	10	3	—	—	—	8
	II	2	—	—	—	—	1
	III	5	2	1	1	—	3
	IV	10	3	1	—	—	5
	V	15	16	9	4	4	25
	Total	42	24	11	5	4	42
Positive specimens (IV and V) in per cent		60	79	91	80	100	71
Secondary staining (restaining) by the SH- method	I	12	4	1	—	—	3
	II	8	6	2	2	1	6
	III	25	13	4	5	3	17
	IV	17	16	10	7	7	16
	V	31	20	18	12	6	51
	Total	93	59	35	26	17	93
Positive specimens (IV and V) in per cent		52	61	80	73	76	72

Classification

Class I. Normal cell picture, negative for malignancy.

Class II. Atypical cytology, no evidence of malignancy.

Class III. Cytology suggestive of, but not conclusive for, malignancy.

Class IV. Cytology strongly suggestive of malignancy.

Class V. Cytology conclusive for malignancy.

The SH-method gave the best result, and cytologically malignant cells were demonstrated in 49% of all cases. Similar results were obtained with the Papanicolaou method, while the AO-method was clearly inferior.

Class I. A quite normal cell picture was observed in five patients, three with squamous cell carcinoma (Nos. 6, 11, 21) and two with anaplastic carcinoma (Nos. 27 and 28).

Class II. The remaining 38 cancer patients upon cytological examination of bronchial secretions had cells with an increased concentration of protein-bound SH-groups and a varying degree of atypia. These abnormalities, however, were not sufficient to provide a suspicion of malignancy in seven of the patients: four with squamous cell carcinoma (Nos. 2, 12, 16, 22), two with

TABLE 16. *Distribution of 43 patients with primary bronchogenic carcinoma with respect to the results of the cytological examination of bronchial secretions using the SH-, AO- and Papanicolaou staining methods*

Classification	Staining method. No. of cases		
	SH	AO	Papanicolaou
I	5	2	11
II	7	2	4
III	10	2	9
IV	6	—	8
V	15	1	11
Total	43	7	43
Positive specimens (IV and V) in per cent	49	14	44

Classification

Class I. Normal cell picture, negative for malignancy.

Class II. Atypical cytology, no evidence of malignancy.

Class III. Cytology suggestive of, but not conclusive for, malignancy.

Class IV. Cytology strongly suggestive of malignancy.

Class V. Cytology conclusive for malignancy.

anaplastic carcinoma (Nos. 31 and 32) and one without histopathological verification but with a clinical diagnosis of cancer (No. 39).

Class III. Suspected malignant cells were recognized in bronchial secretions in ten of the cancer patients: five with squamous cell carcinoma (Nos. 5, 7, 10, 15, 17), two with adenocarcinoma (Nos. 23 and 25), one with anaplastic carcinoma (No. 30) and two without histopathological verification but with a clinical diagnosis of cancer (Nos. 40 and 42).

Classes IV and V. A highly increased concentration of proteinbound SH-groups was demonstrated in atypical cells of bronchial secretions in 21 of the patients: ten with squamous cell carcinoma, two with adenocarcinoma, seven with anaplastic carcinoma and two without histopathological verifications but with a clinical diagnosis of bronchogenic carcinoma.

Cytological examination of bronchial secretions compared to that of sputum. Upon simultaneous sputum examination cytologically malignant or suspected malignant cells were demonstrated in bronchial secretions of all cancer cases with exception of two patients (Nos. 9 and 40). These had atypical cells without a markedly increased concentration of proteinbound SH-groups (Class II).

Upon microscopical examination of bronchial secretions from four patients with a *metastatic pulmonary process*, cytologically malignant cells were ob-

served in two cases (Nos. 110 and 111) with a rectal carcinoma and hypernephroma, respectively. In the other two patients (Nos. 112 and 113) with hypernephroma metastases, however, no suspected or malignant cells could be demonstrated in the bronchial secretions. But upon sputum examination cytologically positive cells could be demonstrated in all four patients.

Bronchial secretions were examined cytologically in two patients with *benign pulmonary tumors*. In one case (No. 126) atypical cells were observed with a certain increase of the SH-concentration. At operation the histopathological examination revealed a hamartoma. In the other patient (No. 128) with a clinical diagnosis of bronchial adenoma without histopathological verification, bronchial secretions contained quite normal cells after staining by the SH-method.

In *other diseases of the respiratory tract* examination by the SH-method presented a quite normal cell picture. However, false positive cancer diagnoses (Class IV) were reported in 1.6%, namely, in two patients with a pulmonary abscess and a bronchopneumonia, respectively. One patient with bronchial asthma without signs of a malignant disease had atypical cells (Class III) in bronchial secretions. Upon examination of the sputum from these patients by the SH-method, SH-rich cells in the asthma case were interpreted as cytologically malignant (Class IV) while cytological signs of malignancy were not present in the remaining two patients.

CONCLUSIONS

Upon the examination of bronchial secretions by the SH-method cytologically malignant cells were demonstrated in 49% and suspected malignant cells in 23% of 43 patients with primary bronchogenic carcinoma. False positive cancer diagnosis were reported in 1.6% of all the 121 patients examined.

2. THE ACRIDINE-ORANGE(AO) METHOD

A. Sputum

Fluorescence microscopy after staining by acridine-orange (AO) was performed in a total of 210 representative sputum specimens from 88 patients (Tables 2 and 3, page 22), i.e. an average of 2.5 specimens per patient. The composition of this material according to the final clinical diagnoses is presented in Table 17, where the figures refer to the number of patients with the actual diagnosis (cf. Table 4, page 23).

Among patients with a *primary bronchogenic carcinoma* the squamous cell type (14 cases) predominated over adenocarcinoma (five cases) and anaplastic or undifferentiated carcinoma (three cases) (see Table 18, page 67).

TABLE 17. 115 clinical diagnoses in 88 patients with cytological examination of sputum and 25 clinical diagnoses in 25 patients with cytological examination of bronchial secretions using the AO-method

Diagnosis	Sputum	Bronchial secretions
A. <i>Malignant neoplastic pulmonary diseases</i>		
Primary bronchogenic carcinoma	23	7
Secondary malignant process (pulmonary metastases)	2	1
B. <i>Other malignant diseases without pulmonary metastases</i>	—	—
C. <i>Benign neoplastic pulmonary diseases</i>	1	—
D. <i>Other diseases of the respiratory tract</i>		
Hemoptysis of uncertain origin	2	1
Sinuitis, bronchitis (acute, chronic)	6	4
Bronchopneumonia, pleuropneumonia, pulmonary abscess	25	5
Pulmonary tuberculosis (active or healed)	13	—
Pulmonary mycosis	2	1
Bronchiectases, pulmonary emphysema	15	2
Bronchial asthma	5	1
Pulmonary atelectasis, middle lobe syndrome	3	1
Silicosis	1	—
Sarcoidosis	2	—
Hemosiderosis	1	—
Exsudative or dry pleuritis, pleural empyema, hematothorax, healed pleuritis	6	1
E. <i>Diseases of the cardiovascular system</i>	5	—
F. <i>Various diseases and unclear diagnoses</i>	3	1
Total	115	25

As with the SH- and the Papanicolaou methods the findings were grouped upon cytological assessment into five classes according to the criteria of malignancy present. In this respect attention was paid partly to the character and intensity of the fluorescence and partly to the morphological appearance of the cells. The results are shown in Table 13 a (page 56), Table 18 (page 67) and in Fig. 52 (page 58). Table 18 also shows the accuracy of the AO-method for the diagnosis of different histological types of primary bronchogenic carcinoma.

Class I. In sputum specimens from the 23 patients with *primary bronchogenic carcinoma* a quite normal cell picture was observed in ten cases. In six of these cases representing squamous cell carcinoma, malignant cells or cells suspected of malignancy were also recognized by SH- or Papanicolaou staining (Nos. 3, 7, 8, 48, 49, 50). In the remaining cases, three patients with adenocarcinoma (Nos. 67, 68, 70) and one with anaplastic carcinoma (No. 47), only

TABLE 18. *Results of cytological examination of sputum using the AO-method in 23 patients with primary bronchogenic carcinoma with respect to the histopathological type*

Histopathological type of carcinoma	No. of patients	Positive specimens (IV and V) in per cent
Squamous cell carcinoma	14	29
Adenocarcinoma	5	40
Anaplastic carcinoma	3	0
Clinical cancer diagnosis	1	(100)
Total	23	30

normal cells were observed in all sputum specimens using these staining methods.

Class II. In the sputum of three patients, one with squamous cell carcinoma and two with anaplastic carcinoma, unclear fluorescence conditions and a slightly abnormal cell morphology were observed (Nos. 5, 27, 28). Malignant or suspected malignant cells were identified, however, using the SH- and Papanicolaou methods.

Class III. In three cases of squamous cell carcinoma suspected malignant cells were recognized by the AO-method. By the two other staining methods, however, cytologically malignant cells were identified in sputum (Nos. 2, 44, 46).

Classes IV and V. The remaining seven cases, four squamous cell carcinomas, two adenocarcinomas and one with the clinical diagnosis of cancer, showed cancer positive sputum cytology with AO-staining and the same result by the SH- and Papanicolaou staining (Nos. 1, 4, 6, 45, 66, 69, 97).

Two patients with *extrapulmonary carcinoma* (Nos. 110 and 114) had metastases in the lungs from a rectal adenocarcinoma and a carcinoma of the papilla of Vater. In these cases malignant cells were identified cytologically in sputum specimens by all the three methods.

Upon the cytological examination of sputum from one patient (No. 127) with a *benign pulmonary tumor* (pulmonary carcinoid) no atypical cells were found by the AO-method, while cells suspected of malignancy were observed by both the SH- and Papanicolaou staining.

Among patients with *other diseases of the respiratory tract* without signs of malignancy there were, after staining by the AO-method as well as by the other methods, some cases with erroneous interpretation of the cytological findings. One patient with sarcoidosis had atypical cells in sputum which were considered malignant by the AO-method (Class V). A very high suspicion of malignancy (Class IV) was expressed in sputum specimens from two pa-

tients with an unspecific bronchopneumonia. These three cases with cytological false cancer positive reports constitute 3.4 % of all the 88 patients examined by the AO-method.

CONCLUSIONS

Upon the examination of sputum by the AO-method cytologically malignant cells were identified in 30% and cells suspected of malignancy in 13% of the 23 cases with primary bronchogenic carcinoma. False positive reports were given in 3.4 % of all the 88 patients examined.

B. *Bronchial secretions*

Fluorescence microscopy of bronchial secretions after staining by acridine-orange (AO) was performed on a total of 27 specimens from 25 patients. In two patients specimens were collected on two different occasions, where the cytological assessment, however, was the same in the first and second specimen. The composition of this small material according to the final clinical diagnoses is shown in Table 17.

Seven patients with *primary bronchogenic carcinoma* were examined by the AO-method: five patients had a squamous cell carcinoma and two had an anaplastic carcinoma. Cytologically malignant cells were identified in 14%. The result of the cytological assessment by the AO-method is presented in Table 16 (page 64) and Fig. 52 (page 58).

Classes I and II. A quite normal cell picture was observed in two patients with a squamous cell carcinoma (Nos. 6 and 7). Slightly atypical cells with a reddish fluorescence of the cytoplasm but without suspicion of malignancy were observed in bronchial secretions from two patients with anaplastic carcinoma (Nos. 27 and 28).

Class III. Cells suspected of malignancy with a moderate reddish fluorescence of the cytoplasm were recognized in bronchial secretions from two patients with a low differentiated squamous cell carcinoma (Nos. 1 and 2).

Class V. Cytologically malignant cells with a red fluorescence of the cytoplasm and a characteristic morphology were identified in bronchial secretions from one case of squamous cell carcinoma of a moderately high differentiation (No. 4). In this patient, however, morphologically atypical cells were also found, where the characteristic red fluorescence of the cytoplasm was absent and only a faint olive-green fluorescence could be demonstrated (Fig. 38).

An intense red fluorescence in both cytoplasm and nucleus was observed in bronchial secretions in one patient (No. 110) with pulmonary carcinosis and a primary carcinoma of the rectum.

In other *non-malignant pulmonary diseases* no suspected or false positive

cancer diagnoses were obtained after cytological examination of bronchial secretions by the AO-method.

Results obtained at the examination of bronchial secretion and sputum agree in all patients, excepting Nos. 1 and 6 with squamous cell carcinoma. Cytologically cancer positive cells were observed in sputum but not in bronchial secretions, which contained normal and suspected malignant cells, respectively.

CONCLUSIONS

Upon the examination of bronchial secretions by the AO-method cytologically malignant cells were demonstrated in 14% and cells suspected of malignancy in 29% of seven patients with primary bronchogenic carcinoma. There were no false positive reports among all the 25 patients examined.

3. THE PAPANICOLAOU METHOD

A. Sputum

The sputum specimens which were assessed after staining by the SH-method were also examined after staining with the Papanicolaou method. Thus, 1,073 specimens containing a representative cell material from 454 patients were examined (Tables 2 and 3, page 22), i.e. an average of 2.5 specimens from each patient. The composition of the material and its comprehension are presented in Chapter II.

Concerning patients with primary bronchogenic carcinoma a maximum of 74% could be diagnosed by the Papanicolaou method (Table 13 a, page 56 and Fig. 52, page 58). However, this was only possible after examination of a great number of specimens from each patient, in some cases up to about 20. With a single specimen practically every second cancer case could be detected, but cytologically examination of two, three or four specimens was of diagnostic significance in about 60%.

Class I. With the Papanicolaou method a quite normal cell picture was observed on cytological examination of sputum from twelve patients with primary bronchogenic carcinoma (11.2%). In five of these cases, Nos. 67, 68, 70, 79 with adenocarcinoma, and case No. 47 with anaplastic carcinoma, the cell findings were normal even with the SH- and AO-methods. In the remaining seven patients with negative Papanicolaou smears suspected or malignant cells were demonstrated by the SH-method.

Class II. Atypical cells were recognized in sputum stained by the Papanicolaou method in nine patients with primary bronchogenic carcinoma (8.4%) where a higher cytological classification was impossible (Nos. 9, 12, 21, 33, 39, 50, 57, 64, 94). However, suspected or malignant cells were recognized

in sputum by the SH-method in all these patients except one (No. 9) who had only certain cells with a slightly increased concentration of proteinbound SH-groups without other signs of malignancy.

Class III. Suspected malignant cells were observed in sputum specimens stained by the Papanicolaou technique in seven patients with primary bronchogenic carcinoma (6.5 %) without that a higher class of assessment was possible from a cytological point of view (Nos. 7, 28, 29, 65, 84, 91). In only one of these cases, No. 65, the SH-method gave inferior results, while sputa from the remaining cases contained suspected or malignant cells.

Classes IV and V. Gravely atypical cells with a very high suspicion of malignancy or signs of definite malignancy were identified in sputa from 79 patients (74 %) with a primary bronchogenic carcinoma after staining with the Papanicolaou method.

In sputum specimens from a case of *primary pulmonary sarcoma* (No. 108) only slightly atypical cells without suspicion of malignancy were recognized, while cytologically malignant cells were demonstrated by the SH-method. In a case of a rhabdomyosarcoma (No. 109), probably primary of the lung, suspected malignant cells were found in sputum with the Papanicolaou method as well as by the SH-method.

Extrapulmonary carcinoma or sarcoma was diagnosed in 23 patients. Metastases were found in one or both lungs in 11 cases (Tables 4, page 23 and 11, page 29). In these patients a normal cell picture was observed only in one patient with hypernephroma, who showed a great number of large and small rounded densities in both lungs on X-ray examination (No. 120). Also with the SH-method only a normal cell picture was present. In one case of liposarcoma (No. 119) atypical cells without signs of malignancy were observed in sputum by the Papanicolaou as well as the SH-method. The same findings occurred in a patient with a carcinoma of the colon and rectum and pulmonary metastases (No. 117). The SH-method showed here a normal cell picture. Suspected malignant cells were demonstrated in sputum by both the Papanicolaou and SH-methods in a patient (No. 116) with a gastric ulcer with malignant changes, adenocarcinomatous in type, and pulmonary metastases. The remaining patients with pulmonary metastases displayed cytologically cancer positive cells in sputum by both the Papanicolaou and SH-methods (Nos. 110, 111, 112, 113, 114, 115, 118).

Of the remaining twelve patients with extrapulmonary malignant processes without signs of metastases of the lungs, nine had a normal cell picture in the sputum. Cells suspected of malignancy were, however, observed in two cases with a primary tumor of the prostate and the thyroid. The patient with carcinoma of the prostate had no pulmonary metastases at the occasion of investigation, but had an active pulmonary tuberculosis together with a

pronounced cardiosclerosis and several acute periods of heart failure. The patient with carcinoma of the thyroid had at the occasion of the cytological examination a hitherto undiagnosed metastasis of the nasal vestibulum, which, of course, can explain the pathological cell findings (Stahle & Wiman, 1962). In the first patient, the SH-staining was quite negative in respect of malignant cells, but definitely malignant cells were demonstrated by the SH-method in the second patient. Malignant cells were also identified in sputum by the Papanicolaou and the SH-methods in one patient with a primary squamous cell carcinoma of the oesophagus penetrating to the trachea.

Numerous gravely atypical cells which, however, appeared singly, were observed in sputa with a high admixture of blood from a patient with acute myeloid *leukemia* and leukemic infiltrations of both lungs (No. 121). They were first interpreted as malignant cells from a squamous cell carcinoma with moderately high differentiation, but corresponded most likely to myeloblasts. Another patient with a chronic lymphatic leukemia without hemoptyses (No. 122) presented in sputum a cell picture with only slight morphological abnormalities without even a slight suspicion of malignancy. The same findings were obtained in two patients suffering from *malignant lymphogranulomatosis* (No. 123) and *myelomatosis* (No. 124). Eventually a case of *mycosis fungoides* showed a quite normal cell morphology in the sputum (No. 125).

Benign pulmonary tumors were diagnosed in five patients who were examined cytologically by the Papanicolaou technique. A quite normal cell picture in sputum was observed in a patient with a hamartoma (No. 126) verified at operation, and in one patient (No. 129) with a clinical diagnosis of bronchial adenoma without a histopathological examination. Another patient (No. 128) also had a clinical diagnosis of bronchial adenoma without a histopathological diagnosis where sputum contained atypical cells, however, without suspicion of malignancy. The same finding was observed in a patient (No. 130), who had over several years an unchanged tumor, the size of an orange, in the right lower lobe, and at the same time a pernicious anemia. One patient with a benign pulmonary tumor and cells suspected of malignancy in the sputum (No. 127) had a pulmonary carcinoid with penetration of the capsula and a certain degree of nuclear polymorphism.

Cytological examination of sputum in patients with *other diseases of the respiratory tract* usually showed a quite normal cell picture or cells with slight morphological abnormalities. In the following 34 cases, however, atypical cells were erroneously interpreted as cytologically malignant or suspected malignant.

Class V. In a 72-year-old man with a long-lasting bronchopneumonia gravely atypical cells were interpreted as cytologically malignant, although no malig-

nant growth within the respiratory tract could be demonstrated using other methods of investigation. In the same way cells with pronounced atypia were interpreted as probably malignant in a 60-year-old man with bronchopneumonia and a clinical suspicion of pulmonary carcinoma.

Class IV. Another nine patients had, upon cytological sputum examination using the Papanicolaou technique, a very high suspicion of malignancy, and the sputum specimens were for this reason interpreted as cytologically positive. No malignant disease was detected in these patients after repeated controls. In five cases the results agreed with the findings with SH-staining, where suspected or malignant cells could be demonstrated, and in one of these cases also with the AO-method. The clinical diagnoses in these patients were the following: pulmonary embolism, bronchial asthma, two bronchopneumonias and one case with unclear clinical and histopathological diagnosis. In the remaining cases, using the SH-method, a normal cell picture or cells with only a slightly increased concentration of proteinbound SH-groups could be observed (Clinical diagnoses: pulmonary embolism, bronchial asthma, pulmonary tuberculosis and intercostal neurinoma).

In relation to the total number of patients examined (454) with a representative cell material in sputum, the above-mentioned eleven patients with a false positive cancer diagnosis after staining with the Papanicolaou technique constituted 2.4 %.

Class III. With the same staining method cells suspected of malignancy were observed in sputum in 23 patients (5 %) without a known malignant disease. The clinical diagnoses in these patients are listed in Table 19, where the same patient may have more than one diagnosis.

CONCLUSIONS

Upon the examination of sputum by the Papanicolaou method cytologically malignant cells were demonstrated in 74 % and cells suspected of malignancy in 7 % of 107 cases with primary bronchogenic carcinoma. False positive diagnoses were reported in 2.4 % of all the 454 patients examined.

B. *Bronchial secretions*

Cytological examination of bronchial secretions by the Papanicolaou method was performed in 126 specimens from 121 patients (Tables 2 and 3, page 22). Specimens containing insufficient or poorly treated material were excluded. In one patient with primary bronchogenic carcinoma (No. 4) bronchial secretions from two different occasions were examined, and cytologically malignant cells were demonstrated in the last specimen, while the first presented only a normal cell picture. Bronchial secretions from two different occasions of bronchoscopy were obtained in one patient and a good

TABLE 19. 40 clinical diagnoses in 23 patients with suspected malignant cells in sputum but without a malignant disease. The Papanicolaou method

Bronchopneumonia, pleuropneumonia,	
pulmonary abscess	16
Pulmonary tuberculosis	3
Allergic diseases	1
Bronchitis, acute or chronic	1
Sarcoidosis	2
Bronchiectases	4
Pulmonary emphysema	1
Pulmonary atelectasis	0
Spontaneous pneumothorax	1
Exsudative pleuritis, pleural empyema,	
healed pleuritis	4
Cardiovascular diseases	2
Hemoptysis	1
Pernicious anemia	1
Various diseases and unclear diagnoses	3
Total	40

agreement was found between the first and second specimens in this case with a nonmalignant pulmonary disease and a normal cell picture. In one patient with bronchopneumonia atypical cells in the first specimen of bronchial secretions were interpreted as being suspected of malignancy, while the other specimen contained atypical cells without a suspicion of malignancy. In two cases of bronchogenic carcinoma there was a varying cytological assessment of the bronchial secretions from the first and second specimens. Regarding this small material no definite conclusions concerning the importance of repeated bronchoscopical specimen collection could be drawn. The comprehension and composition of the material are presented in Chapter II.

Bronchial secretions from 43 patients with a *primary bronchogenic carcinoma* were examined by the Papanicolaou method and cytologically malignant cells were demonstrated in 44% (Table 16, page 64 and Fig. 52, page 58). In the cases where specimens were obtained from different bronchoscopies the highest classification was always used for the compilation of the final results.

Class I. A quite normal cell picture was observed in eleven patients, seven with a squamous cell carcinoma (Nos. 5, 6, 9, 10, 12, 17, 21), three with an anaplastic carcinoma (Nos. 30, 32, 34) and one without histopathological verification (No. 39). Upon examination of sputum by the Papanicolaou method, however, cytologically malignant or suspected malignant cells (Clas-

ses V, IV and III) could be demonstrated in six of these patients. Only in two cases was the cell picture quite normal (Class I).

Class II. Atypical cells without suspicion of malignancy were recognized in bronchial secretions in four patients, one with squamous cell carcinoma (No. 18), one with adenocarcinoma (No. 23) and two with anaplastic carcinoma (Nos. 27 and 31).

Class III. Cells suspected of malignancy in bronchial secretions were demonstrated by the Papanicolaou method in nine patients, seven with a squamous cell carcinoma (Nos. 1, 7, 13, 14, 15, 16, 22), one with anaplastic carcinoma (No. 28) and one without histopathological verification (No. 40).

Classes IV and V. In bronchial secretions from 19 patients out of a total of 43 with primary bronchogenic carcinoma, cytologically cancer positive cells were identified by the Papanicolaou method. At the examination of sputum cytologically cancer positive cells were identified in 15 of these patients and in another patient cells suspected of malignancy were observed using the Papanicolaou method. In the remaining three cases the cytological examination of bronchial secretions was superior to that of sputum.

Among four patients with a *metastatic pulmonary process* (Nos. 110, 111, 112, 113) cytologically positive cells were observed in bronchial secretions in one case (No. 110) where the primary tumor was rectal in origin. In the remaining patients no signs of malignancy appeared. With sputum examination cytologically positive cells were observed in all cases.

In two patients with *benign pulmonary tumors* (Nos. 126 and 128) a cell picture without cytological signs of malignancy was observed by the Papanicolaou method in bronchial secretions.

In patients with *other diseases of the respiratory tract* the cytological examination of bronchial secretions by the Papanicolaou method revealed a cell picture without marked abnormalities. Atypical cells were, however, erroneously interpreted as cytologically malignant (Classes IV and V) in three patients (2.5 %). In one case of bronchial asthma there was no clinical suspicion of cancer. The second patient had six years previously been operated upon for a pulmonary carcinoma of the squamous cell type, and he died four years later of a probable carcinoma of the oesophagus. A third patient with pulmonary tuberculosis and a secondary mycotic infection was operated upon on a suspicion of cancer which, however, could not be verified at the histopathological examination. Upon sputum examination by the Papanicolaou method atypical cells were interpreted as cytologically malignant in the asthma case, while in the remaining two cases no signs of malignancy were observed.

CONCLUSIONS

Upon the examination of bronchial secretions by the Papanicolaou method cytologically malignant cells were demonstrated in 44 % and cells suspected of malignancy in 21 % of 43 patients with primary bronchogenic carcinoma. False positive diagnoses were reported in 2.5 % of all the 121 patients examined.

4. VARIOUS COMBINATIONS OF THE SULFHYDRYL(SH), ACRIDINE-ORANGE(AO) AND PAPANICOLAOU METHODS

Cytological examination of sputum with any one of the methods described, namely, SH-, AO- or Papanicolaou staining, showed that with a sufficiently large number of specimens from each patient the SH- and Papanicolaou techniques were of equal value. The SH-method was, however, the most accurate if only 5 specimens were available. The AO-method was clearly inferior to the other staining methods (see Table 13 b).

In order to assess the value of the different combinations between the staining methods used, a compilation was made in Table 20 of the results obtained from the first sputum specimen of all the patients in the material with primary bronchogenic carcinoma. Staining with all three methods was thus performed on sputum specimens from 23 patients, wherewith the cytological assessment was positive in 38 %. The combination of the Papanicolaou technique and AO-staining gave a positive result of only 28 %. The AO-technique together with the SH-method gave, on the contrary, a better result, in that 41 % positive specimens could be registered with the cytological investigation of the same 23 patients. An even better result, with 49 % cytologically positive specimens, was obtained by combination of the Papanicolaou technique with SH-staining. This was performed on the first sputum specimen from all the 107 patients with primary bronchogenic carcinoma. A comparison between the different combinations and each method by itself, showed, however, that staining for the demonstration of proteinbound SH-groups alone was the most satisfactory, with 54 % positive specimens.

Based upon the combination which gave the best result with only one sputum specimen, namely, SH-staining and the Papanicolaou method, the positive and suspected positive results of the total number of specimens from all 454 patients are presented in Table 21. Herewith special consideration was given to false positive diagnoses, that is, patients who upon cytological examination were placed in one of classes IV or V even though other investigations did not reveal a diagnosis of malignant disease. Missed cancer cases, that is, patients with a histopathologically verified or clinically certain

TABLE 20. *Results of cytological examination of the first sputum specimen from patients with primary bronchogenic carcinoma. Evaluation of various staining methods and combinations*

Method or combination	No. of examined		No. of patients in class					First specimen positive reports in per cent
	Patients	Specimens	I	II	III	IV	V	
SH	107	107	17	6	26	20	38	54
AO	23	23	14	3	—	3	3	26
Papanicolaou	107	107	31	10	19	14	33	44
SH and AO	23	46	21	3	3	6	13	41
Papanicolaou and AO	23	46	24	4	5	1	12	28
SH and Papanicolaou	107	214	48	16	45	34	71	49
SH, AO and Papanicolaou	23	69	31	4	8	4	22	38

Classification

Class I. Normal cell picture, negative for malignancy.

Class II. Atypical cytology, no evidence of malignancy.

Class III. Cytology suggestive of, but not conclusive for, malignancy.

Class IV. Cytology strongly suggestive of malignancy.

Class V. Cytology conclusive for malignancy.

bronchogenic carcinoma without positive cytology in any specimen, were also given special attention. For interpretation of the cytological examination, the relation between false (*F*) and missed (*M*) cancer diagnoses at different class-combinations with both staining methods can be of interest. Variations of *F* and *M* were most easily shown graphically by the straight line

$$y = F \cdot x + M.$$

On the occasions when cytological examination of sputum is performed it is always desirable that the number of false and missed cancer diagnoses are reduced to a minimum, and both *F* and *M* should therefore adopt as low values as possible. But in the above equation, *F* is the tangent of the angle of inclination, and when *F*=0, the line is parallel to the abscissa. *M*, however, gives the point where the line cuts the ordinate, and with *M*=0, passes through the origin. Consequently, the ideal straight line coincides with the *x*-axis.

However, neither false nor missed cancer diagnoses can be completely avoided in diagnostic cytology, and the importance of these vary considerably, depending upon the character of the investigation. If the cytological sputum examination is used as a so-called "search method" (in German: Suchmethode)

to detect lung cancer at an early stage, perhaps already before symptoms and X-ray changes have appeared, then a false positive diagnosis is usually less serious than a missed cancer diagnosis. In this situation the purpose is chiefly to detect cytologically previously unknown cancer cases. Over-diagnosis is not of great importance, as presumed false positive cancer diagnoses can eventually be corrected using other diagnostic procedures. On the other hand, for the final diagnosis, a false positive report has another import and can lead to erroneous surgical or medical treatments with undesirable consequences for the cancerfree patient. With a cytologically missed cancer diagnosis, however, one does not need to pay the same attention in this stage of the disease as several other diagnostic possibilities are usually at one's disposal, including bronchoscopy, special X-ray examinations etc.

This reciprocal evaluation of false and missed cancer diagnoses is illustrated in the graphical presentation of the equation $y = F \cdot x + M$ in Fig. 53. If $x = 0$, then the number of missed diagnoses is indicated on the ordinate. With, for example, $x = 8$, a false positive cancer diagnosis is considered to be 8 times more serious than a missed cancer diagnosis. This situation occurs upon interpretation of the cytological reports for the purpose of a final diagnosis. For early detection of cancer, however, e.g. in health surveys or mass surveys of selected populations, cytological over-diagnosis can be overlooked to a certain extent, the value of x approaches 0 and the importance of missed cancer diagnoses increases to a corresponding degree.

In Fig. 53 the different class-combinations from Table 21 are represented by straight lines with different inclinations (F) and intersections with the ordinate (M). For a given value of x one can therefore easily choose the class-combination that has the greatest relevance in the actual situation. It is found with $x < 1/2$ that the staining for proteinbound SH-groups detected 97 out of 107 patients (91 %) with primary bronchogenic carcinoma, if consideration is taken of both positive and suspected positive reports and the Papanicolaou staining is disregarded (line No. 8; SH: III, IV or V; Papanicolaou: —, that is, the SH-method alone). It is true that a large number of cancer negative patients must undergo additional examinations ($F = 43$), a problem that is, however, of less importance in this situation, and should be balanced against the increased number of patients with a correct cancer diagnosis and the small number of missed cancer diagnoses ($M = 10$). Line No. 7 (SH: — Papanicolaou: III, IV or V, that means the Papanicolaou method alone) has nearly the same inclination ($F = 38$) as that discussed above, and is in this respect of the same value. However, twice as many cancer patients were missed here ($M = 21$), which means that this method can be disregarded in favour of the SH-method when it is a question of early detection of bronchogenic carcinoma, e.g. in mass surveys. If both methods are used together with a combi-

TABLE 21. *Number of cases with primary bronchogenic carcinoma, false (F) and missed (M) cancer diagnoses in certain class combinations using the SH-method and the Papanicolaou technique*

Class combination No. (Line No.)	Highest result classification with		Primary bronchogenic carcinoma	False cancer diagnoses (F)	Missed cancer diagnoses (M)
	SH-method	Papanicolaou method			
1	IV V	IV V	66	6	41
2	IV V	III IV V	69	7	38
3	III IV V	IV V	76	8	31
4	—	IV V	79	11	28
5	IV V	—	81	10	26
6	III IV V	III IV V	82	19	25
7	—	III IV V	86	38	21
8	III IV V	—	97	43	10

Classification

Class I. Normal cell picture, negative for malignancy.

Class II. Atypical cytology, no evidence of malignancy.

Class III. Cytology suggestive of, but not conclusive for, malignancy.

Class IV. Cytology strongly suggestive of malignancy.

Class V. Cytology conclusive for malignancy.

nation of all positive and suspected positive reports, then line No. 6 is obtained, which takes an intermediate position, having a moderate inclination ($F=19$) and a low point of intersection with the y axis ($M=25$). For the value $x < 2\frac{1}{2}$, the SH-method alone is still preferable (line No. 5; SH: IV or V; Papanicolaou: —) if only completely positive reports are registered and Papanicolaou's method is not used. If the combinations are extended so that even suspected positive reports with the SH-method are accepted in the group of positive cases with Papanicolaou's staining, then one can use the line No. 3 for x -values even up to five (SH: III, IV or V; Papanicolaou: IV or V). Within this x -region, however, three lines lie quite close together, namely, besides the two already mentioned Nos. 5 and 3, also No. 4 (SH: —, Papanicolaou: IV or V), where a nearly complete agreement occurs in regard to the inclination ($F=10, 8$ and 11 , respectively). No obvious difference in the significance of the diagnosis with SH-staining and Papanicolaou technique occurs within this region. For $x > 5$ it appears that a combination of the positive results of both staining methods provides the most reliable cytological method of cancer diagnosis. This is represented by line No. 1 in Fig. 53 (SH: IV or V; Papanicolaou: IV or V). It has the smallest inclination of all the lines ($F=6$) but a high y -value ($M=41$) corresponding to very few false positive but many

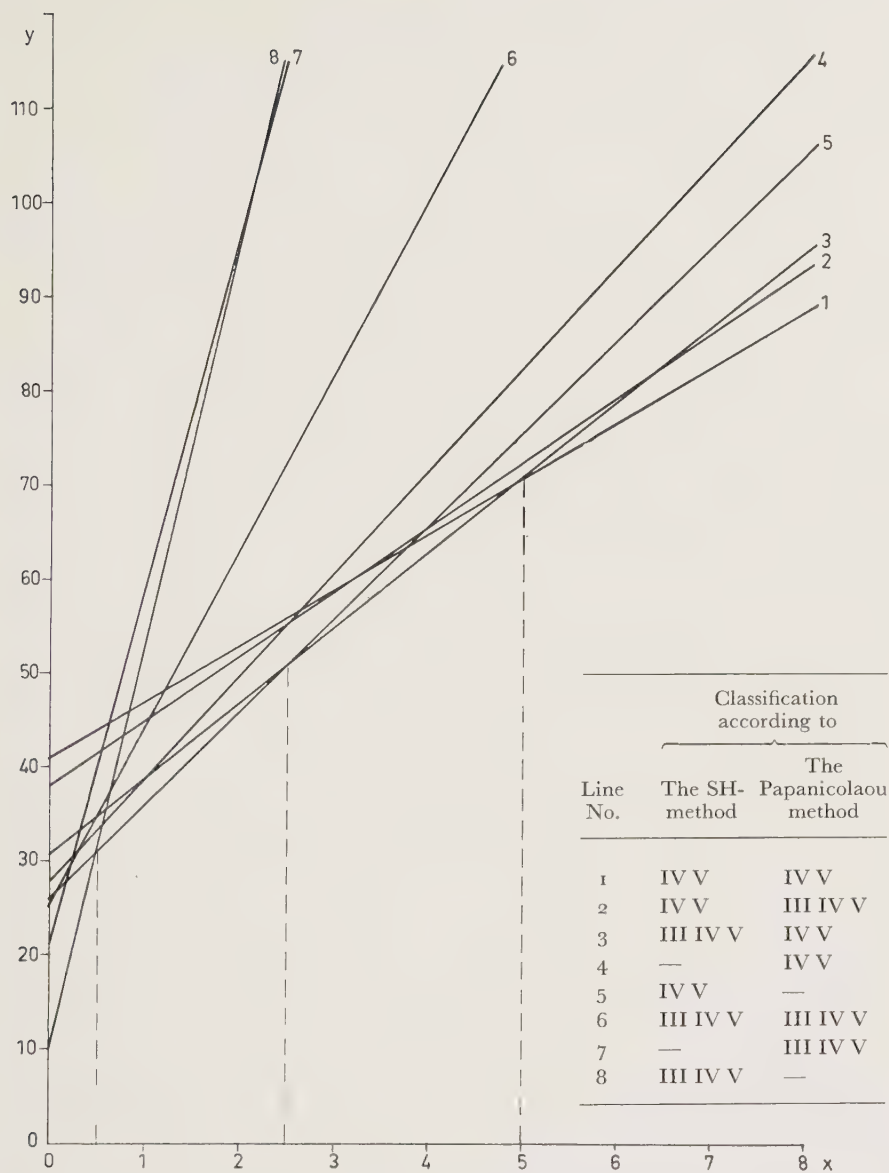


FIG. 53. The relation between false (F) and missed (M) cancer diagnoses in certain class combinations of the SH-method and the Papanicolaou technique expressed by the equation $y = F \cdot x + M$.

missed cancer diagnoses. Line No. 2 (SH: IV or V; Papanicolaou: III, IV or V) has about the same inclination ($F=7$) and intersection with the y -axis ($M=38$) close to that of Line No. 1. For this reason, these cytological class combinations can only be used together with other clinical methods of investigation for the final cancer diagnosis.

CONCLUSIONS

From this analysis it is clear that staining for proteinbound SH-groups is suitable for early diagnosis of primary bronchogenic carcinoma, especially for mass surveys, where the number of new cancer cases detected are of greater importance than any contingent over-diagnosis of false cancer cases that may occur. For the final diagnosis of bronchogenic carcinoma, on the other hand, a combination of the SH-method and Papanicolaou's technique is preferable. Staining of sputum smears with acridine-orange and cytological examination using fluorescence microscopy proved to be inferior to the other staining methods in this study.

DISCUSSION

The greatest possibilities for a successful cytological cancer diagnosis occur when the following conditions are fulfilled: that the growing tumor exfoliates a sufficient number of cells, that these cells are found in the specimen to be examined, that specific laboratory methods are available for identification of the exfoliated cells collected, and also that a knowledgeable person is at hand for the interpretation of the tumor cells present.

I. EXFOLIATION

In normal respiratory epithelium a large cell proliferation continually occurs, which is reflected in a high mitotic index, that is, the proportion of the number of cells in division to the total number of cells (Bertalanffy, 1963). In an animal experimental study Leblond & Walker (1956) using the colchicine method showed that the mitotic index in rats was very high in, among other tissues, the respiratory and alveolar epithelium. Overproduction of cells through mitosis is normally balanced by the desquamation of cells to the lumen, and conditions for the exfoliation of normal cells within the respiratory tract are secured. When malignant tumors occur which invade the bronchial mucous membranes and alveoli the mitotic index, of course, considerably exceeds the normal, and the exfoliation of cells should therefore, at least theoretically, be greater from neoplastic than from normal tissue. It also follows that this exfoliation commences in the earliest developmental stage of the tumor, before macroscopical signs of a neoplasm exist, and in a stage where neither X-ray examination nor bronchoscopy can reveal the nature of the condition. Appel & Bronk (1949) also showed in a study with the experimental production of lung tumors in rabbits that exfoliated cells could be demonstrated in the bronchial secretions in 100% of cases and in 58% of cases in a microscopical stage of the tumor.

Assuming that specimen collection and identification of tumor cells are possible, cytological diagnosis should be the best and surest of the present known methods for the early detection of malignant tumors at a stage where successful therapy is still possible. Histopathological investigations cannot be used in the earliest stages of lung cancer, as localized, macroscopical changes are lacking or are difficult to locate for purposes of biopsy. Cases with positive cytological cancer diagnosis but with negative X-ray result and bronchoscopy were described by Appel & Bronk (1949), Papanicolaou & Koprowska (1951), Koss (1961) and Melamed, Koss & Clifton (1963).

2. SPECIMEN COLLECTION

Material for cytological examination of lung tumors can be obtained by collection of sputum, secretions from bronchoscopy, pleural fluid by thoracentesis and from material secured upon fine needle puncture of lung tumors. The latter two methods of obtaining material require special techniques and instrumentation, and are used mostly as alternative methods for the final diagnosis of lung diseases, and, therefore, fall outside the frame of this subject.

The easiest way to obtain specimens was by collection of *sputum* after the patient had spontaneously coughed up material from the lower respiratory tract. This technique of procuring specimens was simple, quick, without risk and discomfort for the patient and was easily repeated when required. Difficulties might arise in obtaining a specimen representative of the lower respiratory tract containing alveolar cells. The occurrence of ciliated cells were not decisive proof, since these might also originate from the trachea, pharynx, nasal cavity or sinuses. The author's experience showed that the possibilities of obtaining a representative specimen were greatest if collection was made on the morning of the day after bronchoscopy. This observation was also in agreement with the results published by Russel, Neidhardt, Mountain, Griffith & Chang (1963). Several methods of stimulating the cough reflex and secretion in the bronchi were reported in the literature, most of which were based upon the inhalation of aerosols. According to Bickerman, Sproul & Barach (1958) sputum was induced after inhalation of an aerosol, heated to 40°C, of a hypertonic (3, 10 or 15 %) saline solution with 20 % propylene glycol ($\text{CH}_3\text{-CHOH-CH}_2\text{OH}$). Of 180 patients without respiratory tract symptoms 86 % produced representative specimens even though spontaneous expectoration was lacking before inhalation. Similar results were obtained by Berkson & Snider (1960) and Leilop, Garret & Lyons (1961) and others. In the present work, sputum was induced by aerosol inhalation after nebulization of a 10 % saline solution with propylene glycol in a concentration of 20 % without prior heating. Out of 17 patients with non-representative sputum specimens this method gave a satisfactory result from a cytological point of view in 47 % (6 men and 2 women), whilst specimens lacking alveolar cells even after inhalation occurred in 53 % (3 men and 6 women). Of the other methods for sputum induction described in the chapter on specimen collection (p. 31) both intramuscular injection of guajacol-glycerine-ether and oral or intravenous administration of iodine had a good effect on sputum production. In all cases complementary manual thumping, dorsally over both lungs, was resorted to, often in combination with a suitable position of the body for drainage of the actual bronchial segment.

In cases of bronchogenic carcinoma another factor affecting the patient's ability to produce a representative sputum specimen was the extent of obstruction of the bronchus. Total occlusion usually meant completely negative sputum specimens without either tumor or alveolar cells. In the terminal stage of cancer, or in otherwise debilitated or weak patients, there was often a difficulty in obtaining a representative specimen, even after use of the above-mentioned aids.

With *bronchoscopy* the possibilities of obtaining specimens of secretions for cytological examination were always favourable, and alveolar cells as well as cells from all layers of the respiratory epithelium could usually be identified. The specimen was always aspirated prior to biopsy, in order to avoid unnecessary admixture of blood. If there was no visible tumor the suspected bronchial area was irrigated with isotonic saline solution. In order to avoid unnecessary losses of the aspirated material a method was tested in which the material was collected in a container close to the proximal end of the sucker (standard equipment from Pilling & Co., Philadelphia, U.S.A.). By this means adhesion of secretions to the wall of the suction tube was avoided during transport to the cytology laboratory, otherwise the specimens sometimes arrived in an unsatisfactory condition. There were also other practical advantages with this method.

Blind curettage was, as a rule, an unnecessary measure, which produced an altogether too thick a cell preparation and mucous membrane fragments unsuitable for cytological examination. Direct wiping of the suspected mucous membrane area or tumor with a cotton wool or gauze covered instrument was also tried. This technique of collecting cytological specimens within the upper respiratory passages was described by, among others, Stahle & Wi-man (1962). The results of a similar method of specimen collection with bronchoscopy, however, were less satisfactory. The cells were always somewhat deformed and the thickness of the smear preparation varied within wide limits, which made the cytological interpretation unnecessarily difficult.

Under all circumstances, the method of cytological examination of bronchial secretions would appear to provide a more accurate diagnosis than would sputum examination. In a comparison of the two methods of collecting specimens the author's investigations, however, often showed the opposite. A contributory cause might be the premedication of atropine, which reduced the secretions and facilitated the inspection during bronchoscopy. It is possible, however, that a corresponding decrease of exfoliated cells occurred in the specimen collected. In the question of simplicity, rapidity, comfort and risks for the patient, a comparison was, naturally, in favour of the sputum examination. Even if some specimens were not representative of the lower respiratory tract a new specimen could normally be obtained without diffi-

culty whenever required, if necessary after stimulating cough reflex and secretion by the methods already described. Herein really lay the greatest advantage with the sputum method, and the value of repeated specimen collection could scarcely be overrated. A disadvantage, compared with cytological examination of bronchial secretions was, however, that the material to be examined originated from both lungs.

The best results were obtained with sputum cytology, and the cytological examination of the first sputum specimen in this study gave results equal to those obtained with bronchial secretions (Fig. 52, page 58). Similar experiences were reported by Farber, McGrath, Benioff & Rosenthal (1950), who could increase the number of correct, positive cancer diagnoses from 35 % to 90 % if at least 5 sputum specimens were analyzed, whilst cytological examination of bronchial secretions gave only 57 % correct positive diagnoses. Umiker (1960) reported that 30 % of the lung cancer cases in his material could be diagnosed with only one sputum specimen and 96 % with more than five specimens, whilst only 40 % were cancer positive with bronchial secretions. In a study of 24 patients with histopathologically confirmed lung cancer Wiman (1960) was able to diagnose 18 by cytological examination of sputum. Using bronchial secretions, however, only 12 presented cytologically malignant cells. Wiman (1962, 1963) presented results showing that bronchial secretions and the first sputum specimen gave 42 % correct positive cancer diagnoses, whilst the combined results of 5 sputum specimens rose to 87 % correct positive diagnoses. Similar results were reported also by Rome & Olson (1956) and Russel, Neidhardt, Mountain, Griffith & Chang (1963) (see Table 1, page 11).

From a fundamental point of view clinical cytology can be used partly for the early diagnosis of symptomless cancer, and partly for differential diagnosis in patients with suspected cancer. Early diagnostic methods are particularly useful in the screening of symptomfree individuals for health surveys and mass examinations. Here, for obvious reasons, there is no question of using bronchoscopy or other instrumental methods of procuring bronchial secretions. Sputum examination is the method of choice in this situation. However, it is usually necessary to use sputum induction methods in order to secure a representative sample. Using a heated, hypertonic saline solution of propylene glycol a mass survey of symptomfree patients was undertaken at the Memorial Center in New York, which resulted in the diagnosis of one previously undetected carcinoma of the lung in the 643 individuals examined (Koss, 1961). Patients with respiratory tract symptoms, especially productive cough, can normally leave a sputum specimen upon the first visit to the doctor, and the specimen collection does not need to be carried out at a hospital. In this way indications for cytological sputum examinations can be

considerably enlarged, and many patients can be brought under treatment earlier than would otherwise have been possible.

At the hospital the cytodiagnosis of lung cancer is of a different character, due to its use as an alternative or complementary method in cases where a malignant disease is suspected. In order to secure a biopsy specimen all patients with suspected lung cancer must undergo bronchoscopy. Bronchial secretions were therefore aspirated at the same time for cytodiagnosis. With a positive cytological cancer diagnosis there followed also a reasonably certain localization to a definite bronchial segment, if this was not known from other examinations. In cases of advanced carcinoma of the lung, however, sputum diagnosis offered evident advantages as a simple, quick and riskfree method, which without undue inconvenience could be repeated as required.

The cytological interpretation of the exfoliated cells was usually more difficult using bronchial secretions. With conventional staining methods there were the possibility of confusing basal cells, lymphocytes and undifferentiated malignant cells, especially if the latter were of the "oat cell" type. Small, dark, homogeneous nuclei and a sparse cytoplasm characterized all these cell types. Since basal cells, free or in small clusters, were of particularly frequent occurrence in bronchial secretions, the untrained cytologist often met difficulties here.

3. STAINING METHODS AND RESULTS

The possibilities for the identification of malignant cells in a given specimen are related to both exfoliation and specimen collection, and in a hospital material usually about 80% of all cases with bronchogenic carcinoma can be diagnosed by sputum examination (Wiman, 1960; Koss, 1962; and others). For bronchial secretions figures between 20% and 80% were given in the literature (Heckner, 1952; Hjelt, 1953; Umiker, 1960; Wiman, 1960; and others) (Table 1, page 11). In its present form, however, pulmonary cytodiagnosis, based upon morphological criteria, is a very time- and labour-consuming and costly method of investigation. The possibilities for erroneous diagnoses are large, especially for untrained cytologists, and extensive practice and experience with specialized training is required to attain the above-mentioned diagnostic accuracy. For these reasons attempts to simplify and improve cytodiagnosis using cytochemical information are well-grounded.

A. *The acridine-orange(AO) method*

When Friedman (1950) introduced fluorescence microscopy clinical cytology entered a new phase of development. Certain cytochemical data were utilized together with conventional cell morphology for cancer diagnosis. The theo-

retical explanation and technical improvements presented by L. von Bertalanffy, Masin & Masin (1956) made fluorescence microscopy, after staining with acridine-orange (AO), into a practical, quick and simple method. However, serious criticism was directed against this method, among others by Hamperl & Schümmelfeder (1961). These authors hold that the red fluorescence of the malignant cells, which was based upon the binding between ribonucleic acid (RNA) and acridine-orange (AO), did not provide a sufficient ground for the diagnosis of malignancy, in that non-malignant cells were to be found with an increased content of RNA and a red fluorescence. The basic dye substance AO could also combine with mucopolysaccharides and other acidic cell components and produce a red fluorescence, leading to an erroneous impression of malignancy. On the other hand, there were tumors where the RNA concentration did not exceed that of normal cells and the characteristic red fluorescence was absent. Furthermore, the RNA might disappear from the cells because of cytolysis. The interpretation of "naked" nuclei (without cytoplasm) was, apart from the nucleoli, limited to an assessment of the morphology present. The fluorescence intensity, directly proportional to the quantity of AO substance bound in the cell, was dependent upon the concentration and pH of the dye solution. The authors postulated that green or red fluorescence was related to a high respectively a low, degree of polymerisation of the cellular nucleic acids, depending on the pH of the dye solution.

Thus the red fluorescence is by no means specific for cancer and no adequate base for the diagnosis of malignancy. In many situations the investigator is therefore referred to the cellular morphology, which, on the other hand, can be more easily demonstrated with many conventional staining methods, e.g. Papanicolaou's technique. Wied & Mangano (1962) also came to the same conclusion. They found the method to be very sensitive and of importance for certain research investigations, but for practical use it had hardly any advantages over Papanicolaou's technique. Umiker, Pickle & Waite (1959), Koss (1961), Liu (1961), Stahle & Wiman (1962), Wellman, McDermott & Gray (1963) and others also emphasized fundamental deficiencies of the fluorescence method.

In this work the experiences of the authors mentioned above were mostly confirmed. The staining was quick and simple to perform, but afflicted with many practical disadvantages. Smears were fixed in a wet condition immediately after the preparation. Air-dried smears were also used, but these often showed an inferior morphology. Fluorescence microscopy, unfortunately, required an expensive equipment and a special arrangement with a semi-darkened room for adaptation to the small light intensities which occurred. Already after 3-4 hours, however, the fluorescence was impaired and a cor-

rect cytological assessment was impossible. Microphotography was made difficult, in that under the exposures in ultraviolet light the red fluorescence usually vanished within 30–60 seconds. The light intensity was therefore not always sufficient for the long exposure times required for the film qualities available (e.g. Super Anscochrome, Ectachrome High Speed), and only the most intense fluorescing objects could be photographed. Marking of cells suspected of malignancy or with other abnormalities was not possible, as the smears were screened without a cover glass or with a loose-lying one. The possibilities of storage were limited, but the preparations could be restained with the same or other methods.

To judge from my own investigations fluorescence microscopy after staining with acridine-orange (AO) was an unreliable method, inferior both to the Papanicolaou and SH-method (Table 13 *b*, page 57). Typing of malignant cells was not always possible, as the degree of keratinization was unclear in squamous cell carcinomas. The number of undetected cancer cases in the sputum material was 16 out of 23. The best results were obtained with pulmonary adenocarcinoma, where two out of five cases could be detected using the AO-method. The false positive cancer diagnoses constituted 3.4 % or twice as much as with the SH-method. For all 23 cases of primary bronchogenic carcinoma only 30 % correct positive cancer diagnoses were obtained using the AO-method. With a combination of the Papanicolaou method the result was 28 %, and with the SH-method 41 %, whilst with all three methods combined a 38 % correct diagnosis of malignancy was achieved using the first sputum specimen (Table 20, page 76).

The use of the AO-technique as a routine method in pulmonary cyto-diagnosis was questionable on theoretical and practical grounds, and a combination with the Papanicolaou or SH-method or both, is recommended. It would seem, however, that the AO-method can be used in research work, especially concerning the distribution of nucleic acids.

B. The sulphydryl(SH) method

SH-groups are of fundamental importance for cell division, metabolism and growth in normal and neoplastic tissues. They occur in the organism mainly in the form of glutathione and cysteine, where the easily reacting SH-groups can freely combine with other substances; and bound to proteins, where they may serve as bonds between the polypeptide chains. Barnett & Seligman (1952) synthesized a disulphide (DDD, page 19) that was specifically reduced by proteinbound SH-groups and to which was coupled an azodye so that a colored end product was formed. Freely reacting SH-groups, in the form of glutathione and cysteine, were first extracted, however, with trichloroacetic

acid dissolved in ethanol. At the same time a powerful protein denaturation occurred and a maximum number of SH-groups were available to react with DDD. Improvements introduced by Bahr (1957) made it possible to perform semi-quantitative microphotometric determinations of proteinbound SH-groups in both sections and smears (Moberger & Bahr, 1957, 1958; Sandritter & Krygier, 1959 and Caspersson & Révész, 1963).

Since the relationship between SH-groups and cancer found support in a number of biochemical and histochemical studies, it was natural to study proteinbound SH-groups in exfoliated cells from different body secretions in order to obtain information of both theoretical and practical value. Preliminary investigations in this field were performed by Wiman (1959, 1960, 1962, 1963) and Stahle & Wiman (1962), studying secretions from the respiratory tract. *These investigations, based upon Bahr's technique, demonstrated an increased concentration of proteinbound SH-groups in certain normal epithelial cells and in cells exfoliated from malignant tumors*, a condition which could be utilized in cancer diagnosis. In principle, the results of my own cytochemical investigations of exfoliated cells from the respiratory tract usually corresponded to other biochemical and histochemical studies on normal tissue and malignant tumors.

Compared to Bahr's technique for sections the author's method for staining smears differed in some respects. All the preparations in this work were fixed for 24 hours in trichloroacetic acid and ethanol. A special study with fixation times varying from 10 minutes to several days did not, however, show any difference in the staining quality between the short and long fixation time. Sandritter & Krygier (1959) also considered a 10-minute fixation sufficient in their study of proteinbound SH-groups in HeLa cells using slide preparations. The time for the last staining step using Fast Black Salt K was adjusted in accordance with the varying thickness of the smears, usually to between 2–5 minutes. Dehydration in ethanol and xylene was preferred to that in acetone, which caused difficulties in mounting.

After restaining the cells appeared in a single color of varying intensity, but with shades of violet or brown. There was no need to change the principles of cytological interpretation, and the results agreed with that of the primary SH-staining.

For the cytological assessment of smears stained by the SH-method a normal light microscope without special arrangements was sufficient. Screening was carried out in the same way as with conventional staining methods. A lower magnification, however, could be used, which reduced the screening time to 2–3 minutes per smear. All cells appeared in a single color scale from the violet or brown, SH-rich, malignant cells to the pale shadows of the normal cells of the respiratory tract and blood cells. The training should be the same

as for a Papanicolaou screener, but the training time could be considerably reduced.

In the cells exfoliated from the epithelium of the upper respiratory tract a moderately increased SH-reaction was obtained, which was more pronounced in the basal than in the superficial cells, an observation which agreed with that made in a histochemical study by Barnett (1952-1953). In the respiratory epithelium, too, the concentration of proteinbound SH-groups was higher in exfoliated basal cells than in the superficial ciliated cells.

The secretion vacuoles of goblet cells were SH-negative. In regard to the alveolar cells a great variation of the SH-concentration was observed, which may partly be explained by the type of phagocytized material (see page 44).

Exfoliated cells with an SH-reaction more marked than normal were observed in patients with certain allergic conditions of the respiratory tract. In one patient, for example, with bronchial asthma, cells were found with a moderately increased concentration of proteinbound SH-groups, leading to a false cancer diagnosis. Specimens of sputum and bronchial secretions contained cell clusters reminiscent of those present in "oat cell" carcinoma. Of other patients with allergic diseases, three with eosinophilic infiltrations of the lung (Löffler's syndrome) had a marked SH-reaction in several specimens, which in two cases led to the diagnosis of suspected malignancy. One patient suffering from panarteritis nodosa with pulmonary lesions had several specimens containing cells with an increased SH-concentration, while one patient with allergic rhinitis had an entirely normal cell picture. The concentration of SH-groups in different organs in patients with allergic conditions is still not fully investigated, and no satisfactory explanation of the above-mentioned observations can be given at present.

In other diseases cells with a moderately increased concentration of proteinbound SH-groups was occasionally observed. A clear non-malignant morphology in these cases, however, excluded any suspicion of malignancy. This happened in some patients with benign pulmonary tumors and pernicious anemia and in cases of squamous metaplasia. Characteristic morphological changes of exfoliated epithelial cells in patients suffering from pernicious anemia were described by Graham & Rheault (1954) and Boddington (1959).

As with other cytodiagnostic staining methods there were also false positive reports with the SH-staining, limited to 1.7% in sputum cytodiagnosis and 1.6% in examination of bronchial secretions. Patients with false positive cancer diagnosis and associated problems were discussed in Chapter VI. These serious disadvantages might be reduced if detailed clinical information is available and a careful morphological screening is performed of the SH-stained smears or by combination with other staining methods. No clinical information, however, was available in this work, with a few exceptions,

while morphological screening of the same specimen was always performed with both the SH- and the Papanicolaou techniques. Combination with Papanicolaou's technique was preferred to that with the AO-method (see Chapter VI).

A certain difference in the staining reaction was obtained in the exfoliated cells from different types of cancer. The highest concentration of protein-bound SH-groups was observed in undifferentiated or low differentiated forms of carcinoma and in squamous cell carcinoma. Information concerning keratinization was unobtainable, but highly differentiated forms of squamous cell carcinoma usually presented a characteristic morphology distinguished by bizarre nuclear forms, e.g. "tadpoles", fairly ample cytoplasm and infrequent cell clusters. Highly differentiated adenocarcinomas exfoliated cells which sometimes appeared pale because of SH-negative secretion vacuoles. This was true also in bronchiolar carcinoma (alveolar cell carcinoma), where the exfoliated cells had a reticulated and vacuolated cytoplasm. Cytological typing with the SH-method was therefore possible to nearly the same extent as with the Papanicolaou method.

As with the AO- and Papanicolaou methods the results with SH-staining were significantly improved if the specimen collection was repeated. With the first sputum specimen or bronchial secretions about 50% of the cancer cases could be detected, while over 80% were diagnosed using three specimens. Further collection of specimens did not provide any significant improvement of the diagnostic results with the SH-method (Table 13 *a*, page 56, and Fig. 52, page 58). Neither did combinations with the AO- or Papanicolaou methods succeed in raising the number of correct positive cancer diagnoses (Table 20, page 76). However, a combination of the SH- and Papanicolaou methods was preferred in certain diagnostic situations (Fig. 53, page 79). As a method for final diagnosis of bronchogenic carcinoma cytological examination of sputum and bronchial secretions was of outstanding importance used together with biopsy, X-ray examinations and other clinical and laboratory investigations. In this situation a false positive cancer diagnosis was assigned great significance. With reference to the statistical analysis in Chapter VI: 4 and Fig. 53 (page 79) it was clear that a combined investigation with the SH- and Papanicolaou methods was desirable where the classes IV and V in both methods were used as a basis of evaluation. Possibly, notice should also be given to suspected positive cancer diagnoses (class III) with the SH-method. If the cytological reports were interpreted in this way, then false and missed cancer diagnoses were avoided to the greatest extent in the differential diagnostic work.

If it is assumed, however, that the investigation is to be used as a cancer detection method and to provide the basis for continued laboratory tests and

clinical investigations, then the first task is to discover as many new cases of lung cancer as possible. The number of missed positive diagnoses must be reduced to a minimum. In this regard the cytological examination of bronchial secretions cannot be used for practical reasons. False positive sputum diagnoses may cause unnecessary anxiety in the patient and give additional trouble through further examinations and extended observation time caused by the result of the investigation. From the diagnostic point of view, however, false positive reports are of less importance, and they should be evaluated together with the results of other examinations. In this situation the SH-method alone gave the best results due to the fact that the number of correct positive cancer diagnoses reached a maximum if the classes V and IV, and even cases suggestive of malignancy in class III, were taken into consideration. The staining qualities of the SH-method may also provide a possible base for automatic screening in the cytoanalyzer according to Bostrom, Tolles & Spencer (1962). A successful development of this idea would release much of the effort which is now spent on screening normal smears.

C. *The Papanicolaou method*

The aim with Papanicolaou's staining technique was originally to study cyclic variations in the vaginal mucous membrane of experimental animals under hormonal influences. However, the method achieved its greatest importance in the diagnosis of uterine cancer, and later also in other cancer forms exfoliating cells. Even if the method has a more or less well-defined cytochemical basis its main purpose is to provide a detailed morphologic picture of the exfoliated cells. In this regard Papanicolaou was very successful, and the stained cells develop a beautiful polychromatic color scale with a good transparence. As a basic, routine method for exfoliative cytology it is accepted in the cytological laboratories throughout the world. However, in many regards the May-Grünwald-Giemsa staining method serves the same purpose, especially for pleural and ascitic fluids, when histopathologic technique according to Wihman (1948) or Silverstolpe (1948) cannot be used. A comparison is, naturally, dependent upon the test material used, but with respiratory tract secretions the morphology of the cell nucleus appears just as distinct, or even more so, with the May-Grünwald-Giemsa staining. Papanicolaou's technique gives, however, a better cytoplasmic staining with improved transparency in clusters or aggregates of cells (Stahle & Wiman, 1962). Upon typing of cells exfoliated from a squamous cell carcinoma Papanicolaou's method is hardly exceeded by any other staining method including the May-Grünwald-Giemsa, AO- and SH-methods. Grunze (1956) suggested a May-Grünwald-Giemsa staining modified according to Pappenheim for a quick diagnosis; a preparation with a sufficient morphology being obtained

in 7–10 minutes. The difference between the various morphological staining methods is usually quite small, and often the most suitable method is a question of the cytologist's personal experience and choice (Grunze, 1961).

Upon the microscopical assessment the Papanicolaou method is more time-consuming than both the AO- and SH-methods and screening of a normal smear usually takes 5–10 minutes. The polychromasia and morphological details are often unnecessarily distracting, time wasting and tiring for the investigator.

The results obtained in this work after staining with Papanicolaou's method corresponded mostly to those found in other similar materials (Table 1, page 11). Upon the cytological examination of bronchial secretion the results of 47% correct positive cancer diagnoses occupied an intermediate position between, for example, Heckner's 20% and Hjelt's 79.5%. It was the author's impression that bronchial secretion in general offered greater difficulties than sputum, especially concerning cells from a low differentiated bronchial carcinoma, which were not easy to distinguish from basal or intermediate cells of the deep layers of the bronchial mucosa.

As with the AO- and SH-methods the diagnostic accuracy using the Papanicolaou method was increased with the first three sputum specimens. In many patients, however, the number of specimens examined was enlarged to between 10 and 20, whereupon the results were considerably improved (Table 13 *a*, page 56; Fig. 52, page 58). However, the results never exceeded those obtained by the SH-method. With the Papanicolaou method alone, 2.4% false positive cancer diagnoses were reported upon sputum examination and 2.2% using bronchial secretions. Combination with the AO-method did not improve the sputum diagnostic results, while, on the other hand, the Papanicolaou and SH-staining of different preparations from the same specimen led to an increased number of cytologically diagnosed cases of lung cancer. For differential diagnostic use, where the significance of a false positive cancer diagnosis was of great importance, the difference between the Papanicolaou and SH-methods was very small. As a method for cancer detection in mass surveys the difference was slight in regard to the number of false positive cancer diagnoses (see Chapter VI: 4 and Table 13 *b*, page 57).

SUMMARY

The purpose of this investigation was to study the presence of proteinbound sulfhydryl(SH) groups in exfoliated cells found in sputum and bronchial secretions, and to try out this method in the cytodiagnosis of bronchogenic carcinoma. The Bahr method for the histochemical demonstration of protein-bound SH-groups was modified and applied to smears of sputum and bronchial secretions. The results of this cytological examination were compared to the results obtained by the Papanicolaou staining technique and, on a small material, by the acridine-orange(AO) fluorescence method. All results were correlated to the histopathological or clinical diagnosis.

1,073 sputum specimens from 454 patients with diseases of the lungs or the respiratory tract were examined by the SH- and Papanicolaou methods. The cytological examination was performed on a clinical suspicion of malignancy. 107 patients had a primary bronchogenic carcinoma and 11 patients had pulmonary metastases from an extrapulmonary malignant tumor. 210 sputum specimens from 88 patients, 23 of which had a primary bronchogenic carcinoma, were examined using the AO-method.

In sputum smears stained by the SH-method all cells appeared in a violet or brown color of a varying intensity, directly proportional to the concentration of proteinbound SH-groups. Normal cells exfoliated from the squamous, respiratory and alveolar epithelium of the respiratory tract, as well as cells originating from the circulating blood, usually presented a slight SH-reaction. Cytologically malignant cells, however, exhibited a marked SH-reaction, which made them easy to identify upon microscopical screening. The differences of the SH-content were determined by microphotometric transmission measurements in some selected cases.

According to the SH-concentration and gross morphology the cells were classified in conformity with the Papanicolaou system for cytodiagnosis of malignancy. The results of this investigation showed a cytologically positive diagnosis of malignancy in maximally 83 % of the cancer cases by the SH-method and 74 % by the Papanicolaou method. The difference was, however, statistically significant only if five specimens were investigated. A result of 50 % correct positive diagnoses was never exceeded by the AO-method. False positive cancer diagnoses were reported in 1.7 % of cases examined by the SH-method and in 2.4 % and 3.4 % by the Papanicolaou and AO-methods, respectively.

In order to detect cytologically a maximal number of cancer patients early in the course of the disease, when a missed cancer diagnosis was of great importance, the SH-method afforded the best result. For the final diagnosis of pulmonary malignancy, when the cytodiagnosis was used as a complementary or alternative method, the combined results of the SH- and Papanicolaou methods were preferable. In this way the number of false positive reports were considerably reduced.

Several sputum specimens collected at different occasions increased the diagnostic accuracy in all the staining methods. The diagnostic optimum, however, was usually reached with three specimens.

126 specimens of bronchial secretions from 121 patients were examined cytologically by both SH- and Papanicolaou methods. 43 patients had a primary bronchogenic carcinoma and four had pulmonary metastases from an extrapulmonary malignant tumor. 27 specimens of bronchial secretions from 25 patients, seven of whom had a primary bronchogenic carcinoma, were examined by the AO-method.

Upon SH-staining of bronchial secretions the normal exfoliated cells usually presented a slight SH-reaction, and the cytologically malignant cells a marked SH-reaction. These results were in conformity with those obtained upon sputum investigation. Cytologically malignant cells were identified in 49% of the cancer cases by the SH-method and in 44% by the Papanicolaou method, which agreed with the results obtained by the examination of the first sputum specimen. One out of seven cancer patients was diagnosed by the AO-method. False positive cancer diagnoses were reported by the SH- and Papanicolaou methods in 1.6% and 2.5%, respectively. No false positive reports were obtained by the AO-method.

FINAL CONCLUSIONS

The investigations showed that of the exfoliated cells found in sputum and bronchial secretions, the normal usually had a low and the malignant a high concentration of proteinbound SH-groups. This was utilized in the cytodiagnosis of primary bronchogenic carcinoma achieving at least the same accuracy as by the Papanicolaou method. In certain situations the SH-method was more accurate as compared to the conventional staining techniques. Of all methods investigated the acridine-orange fluorescence technique was found to be the least accurate and should be used with caution in routine cytodiagnosis.

ZUSAMMENFASSUNG

Der Zweck dieser Untersuchung war, das Vorkommen proteingebundener Sulfhydryl(SH)-Gruppen in exfoliierten Zellen des Sputums und in aspiriertem Bronchialsekret zu studieren sowie diese Methode in der Zytodiagnostik des Bronchialkarzinoms zu prüfen. Die Bahrsche Methode, mit der proteingebundene SH-Gruppen histochemisch nachgewiesen werden können, wurde für die Färbung von Ausstrichpräparaten des Sputums und des Bronchialsekretes modifiziert. Die Resultate dieser zytodiagnostischen Untersuchung wurden mit Resultaten verglichen, die durch Färbung nach der Papanicolaou'schen Technik erhalten wurden und mit einem kleineren Material unter Verwendung der Akridinorange (AO)-Fluoreszenzmikroskopischen Methode. Alle Resultate wurden mit der histopathologischen oder klinischen Diagnose korreliert.

1073 Sputumproben von 454 Patienten mit Krankheiten der Lunge oder des Respirationstraktus wurden mit der SH-Methode und nach Papanicolaou untersucht. Die zytologische Untersuchung war immer durch klinischen Malignitätsverdacht veranlasst. 107 Patienten hatten ein primäres Bronchialkarzinom, und 11 Patienten hatten Lungenmetastasen eines extrapulmonalen malignen Tumors. 210 Sputumproben von 88 Patienten, von denen 23 ein primäres Bronchialkarzinom hatten, wurden nach der AO-Methode untersucht.

In Sputumausstrichen, die nach der SH-Methode gefärbt wurden, erschienen alle Zellen in violetter und brauner Farbe verschiedener Intensität, direkt proportional der Konzentration der proteingebundenen SH-Gruppen. Normale, von Platten-, Zylinder- und Alveolarepithel exfoliierte Zellen, sowie Zellen aus dem zirkulierenden Blute, gaben gewöhnlich eine schwache SH-Reaktion. Zytologisch maligne Zellen entwickelten dagegen eine starke SH-Reaktion, wodurch sie leicht im Mikroskop identifiziert werden konnten. Der Unterschied des SH-Gehaltes wurde in einigen gesonderten Fällen durch mikrophotometrische Transmissionsmessungen bestimmt.

Mit Hilfe der SH-Konzentration und der zytomorphologischen Hauptmerkmale wurden die Zellen in Übereinstimmung mit dem Papanicolaou'schen System für die zytologische Malignitätsdiagnostik klassifiziert. Die Resultate dieser Untersuchung zeigten eine zytologisch positive Malignitätsdiagnose bei maximal 83% der Karzinomfälle mit der SH-Methode und bei 74% mit der Papanicolaou'schen Methode. Der Unterschied war jedoch statistisch nur dann

signifikant, wenn fünf Sputumproben untersucht wurden. Bei Verwendung der AO-Methode wurde ein Resultat von 50 % korrekten positiven Karzinomdiagnosen niemals überschritten. Falsch positive Karzinomdiagnosen kamen bei Verwendung der SH-Methode in 1,7 % der untersuchten Fälle vor, während das Verhältnis bei der Papanicolaouschen Technik und der AO-Methode 2,4 % respektive 3,4 % betrug.

Im Bestreben, zytologisch eine maximale Anzahl von Karzinompatienten frühzeitig im Krankheitsverlauf zu entdecken, wobei die grösste Bedeutung einer verfehlten Karzinomdiagnose zukommt, hat die SH-Methode das beste Resultat ergeben. Für die endgültige Diagnose von Malignität in Lunge und Bronchien, wobei die Zytodiagnostik als komplettierende oder alternative Methode verwendet wurde, war die Kombination der SH-Methode mit der Papanicolaouschen Technik vorzuziehen. Auf diese Weise konnte die Anzahl der falsch positiven Resultate wesentlich reduziert werden.

Die diagnostische Zuverlässigkeit aller Färbungsmethoden wurde durch mehrere zu verschiedenen Zeitpunkten gewonnenen Sputumproben erhöht. Das diagnostische Optimum wurde gewöhnlich mit drei Proben erreicht.

126 Bronchialsekretproben von 121 Patienten wurden zytologisch sowohl nach der SH-Methode wie auch nach Papanicolaou untersucht. 43 Patienten hatten ein primäres Bronchialkarzinom und 4 hatten Lungenmetastasen eines extrapulmonalen malignen Tumors. 27 Bronchialsekretproben von 25 Patienten, von welchen 7 ein primäres Bronchialkarzinom hatten, wurden nach der AO-Methode untersucht.

Bei Färbung des Bronchialsekretes mit der SH-Methode zeigten normale exfoliierte Zellen gewöhnlich eine schwache SH-Reaktion, wogegen die zytologisch malignen Zellen eine starke SH-Reaktion entwickelten. Diese Farbreaktionen stimmen mit denjenigen des Sputums überein. Zytologisch maligne Zellen konnten bei 49 % der Karzinomfälle mit der SH-Methode identifiziert werden und bei 44 % nach Papanicolaou. Dies stimmte mit den Resultaten überein, die mit einer einzigen Sputumuntersuchung erhalten wurden. Von 7 Karzinompatienten wurde einer nach der AO-Methode diagnostiziert. Falsch positive Karzinomdiagnosen kamen bei der SH-Methode und der Papanicolaouschen Technik bei 1,6 % respektive 2,5 % vor. Mit der AO-Methode wurden keine falsch positiven Resultate erhalten.

SCHLUSSEFOLGERUNG

Die Untersuchungen haben gezeigt, dass von den exfoliierten Zellen des Sputums und des Bronchialsekretes die normalen Zellen gewöhnlich eine niedrige und die malignen eine hohe Konzentration proteingebundener SH-Gruppen enthalten. Dies wurde für die Zytodiagnostik des primären Bronchialkarzinoms ausgenutzt. Die dabei erzielte Zuverlässigkeit war mindestens

ebenso gross, wie bei der Technik nach Papanicolaou. Unter gewissen Umständen erwies sich die SH-Methode im Vergleich mit konventionellen Färbungsmethoden als zuverlässiger. Von allen untersuchten Methoden hat sich die Akridinorange-Fluoreszenztechnik als am wenigsten zuverlässig erwiesen und sollte deshalb nur mit gebührender Vorsicht in der Zytodiagnostik verwendet werden.

RESUMEN

El propósito de la presente investigación es el estudio de la presencia de grupos de proteínas unidas a grupos sulfidrilos(SH) en las células de exfoliación encontradas en esputos y secreciones bronquiales, al tiempo que se intenta usar éste método en el diagnóstico del carcinoma broncogénico. El método de Bahr, para la demostración histoquímica de la proteína unida en grupos SH, fué modificada y aplicada a frotis de esputos y secreciones bronquiales. Los resultados obtenidos por éste método de examen citodiagnóstico, fueron comparados con los obtenidos con la técnica de coloración de Papanicolaou y en una serie muy reducida, por el método de la fluoreceína acridina anaranjada (AO). Todos los resultados fueron correlacionados con los diagnósticos clínicos o histopatológicos.

Se examinaron 1073 muestras de esputos de 454 pacientes con enfermedades del sistema respiratorio, por los métodos del SH y de Papanicolaou. Los exámenes citológicos se efectuaron por la sospecha clínica de malignidad. En 107 pacientes se encontró un carcinoma broncogénico y en 11 pacientes, metástasis pulmonares de tumores malignos extrapulmonares. 210 muestras de esputos de 88 pacientes, 23 de los cuales tenían un carcinoma broncogénico primario, fueron examinados usando la técnica de AO.

Las láminas que fueron coloreadas por el método del SH mostraron todas las células teñidas en color marrón o violeta de intensidad variable, directamente proporcional a la concentración en ellas, de proteína unida al grupo SH. Células de descamación normal del epitelio escamoso del árbol respiratorio, bronquial o alveolar, así como células de sangre circulante, usualmente presentaron una débil reacción SH. Células citológicamente malignas, mostraron una reacción SH muy fuerte, lo cual facilitó su identificación en exámenes microscópicos. La diferencia en el contenido del grupo SH, fué determinado por la medida de transmisión microfotométrica en casos seleccionados.

Según la concentración SH y su morfología, las células fueron clasificadas de acuerdo al sistema de Papanicolaou para el citodiagnóstico de malignidad. Con ésta investigación fué posible demostrar un diagnóstico positivo de malignidad citológica en un máximo de 83 % en los casos de carcinoma por el método SH y en 74 % con Papanicolaou. La diferencia sin embargo, fué estadísticamente significativa si se examinaron cinco muestras únicamente. El resultado del 50 % de diagnóstico correcto en casos positivos, no fué sobrepasado por el método AO. Resultados positivos falsos fueron reportados en

1,7% en los casos examinados por el método SH y en 2,4% y 3,4% por el método de Papanicolaou respectivamente.

Con el método SH, se obtuvieron los mejores resultados en la detección citológica del mayor número de pacientes con cáncer, en los primeros estadios de la enfermedad. Para el diagnóstico final de malignidad pulmonar, cuando un diagnóstico citológico fué usado como complemento o como método de alternativa, los métodos combinados de Papanicolaou y de SH, fueron preferidos. En ésta forma el número de falsas positivities fué considerablemente reducido.

La colección de varias muestras de esputos en diferentes ocasiones aumenta la seguridad del diagnóstico con los métodos de coloración. El óptimo de seguridad en el diagnóstico se obtuvo generalmente con tres muestras.

126 muestras de secreciones bronquiales de 121 pacientes fueron examinadas citológicamente por ambos métodos de Papanicolaou y de SH. 43 pacientes tenían un carcinoma bronquial primario y 4, una metástasis pulmonar de un tumor maligno extrapulmonar. 27 muestras de secreciones bronquiales de 25 pacientes de los cuales, 7 tenían un carcinoma bronquial primario fueron examinados por el método AO.

Al efectuar las coloraciones de las secreciones bronquiales, las células de descamación mostraron una reacción débil en tanto que las malignas una reacción SH muy fuerte. Estos resultados estuvieron de acuerdo con los obtenidos en la investigación de esputos. Las células citológicamente malignas fueron identificadas en el 49% de los cánceres por el método SH y en el 44% por el Papanicolaou, lo cual concuerda con los resultados obtenidos al examinar las primeras muestras de esputos. Uno de cada siete pacientes con cáncer, fué diagnosticado con el método de AO. Diagnósticos positivos falsos de cáncer, fueron reportados por los métodos de SH y Papanicolaou en el 1,6% y 2,5% respectivamente. No se obtuvieron positivities falsas con el método AO.

CONCLUSIONES

La investigación mostró que las células de descamación encontradas en esputos y en secreciones bronquiales mostraron una baja concentración de proteína unida al grupo SH en la células normales y en cambio, una alta concentración en las células malignas. Esta propiedad fué utilizada para el citodiagnóstico de carcinoma primario broncogénico por los menos con la misma seguridad que con el método de Papanicolaou. En algunas condiciones el método SH fué más exacto si se compara con las técnicas de coloración corrientes. De todos los métodos estudiados, el de la acridina fluoreceína anaranjada demostró ser el menos exacto y debe ser empleado con reservas en el citodiagnóstico de rutina.

RÉSUMÉ

Le but de ces recherches a été d'étudier la présence des groupes sulfhydriles (SH) protéiques, dans des cellules exfoliées obtenues de crachats et de sécrétions aspirées pendant la bronchoscopie, et aussi d'appliquer cette méthode pour le cyto-diagnostic de cancer des bronches. Le procédé histo-chimique de Bahr, démontrant les groupes SH protéiques, fut modifié et employé pour colorer des frottis de crachats et de sécrétions bronchiales. Le résultat de cet examen cytodiagnostique fut comparé aux résultats obtenus par la méthode de coloration de Papanicolaou, et sur un matériel moins étendu, par la technique de fluorescence d'acridine-orange(AO). Tous les résultats obtenus étaient en corrélation avec un diagnostic histopathologique ou clinique.

1073 prélèvements de crachats de 454 malades avec des affections pulmonaires ou aux voies respiratoires, furent examinés par les méthodes SH et de Papanicolaou. L'examen cytologique était toujours effectué au moindre soupçon clinique de malignité. 107 malades avaient un cancer primitif aux bronches, et 11 malades avaient des métastases pulmonaires d'une tumeur maligne extrapulmonaire. 210 prélèvements de crachats de 88 malades, dont 23 avaient un cancer primitif des bronches, furent examinés par la méthode AO.

D'un frottis de crachat coloré par la méthode SH, toutes les cellules apparurent de couleurs violette ou brune d'intensité variable, suivant la concentration des groupes SH protéiques. Les cellules normales exfoliées de l'épithélium lamellé, cylindrique et alvéolaire, dans les voies respiratoires, ainsi que les cellules provenant du sang, donnèrent en général une réaction SH assez pâle. Mais les cellules malignes au point de vue cytologique donnèrent une réaction marquante, ce qui facilitait leur identification au microscope. La différence de concentration SH fut mesurée par transmission microphotométrique dans quelques cas sélectionnés.

Suivant la teneur en SH protéique et les caractéristiques principales cytomorphologiques, les cellules furent classifiées d'après le système de Papanicolaou pour le cyto-diagnostic de malignité. On pouvait constater que le diagnostic cytologique de malignité était positif au maximum dans 83 % des cas de cancer par la méthode SH, et dans 74 % des cas par la méthode de Papanicolaou. Statistiquement cette différence n'était pas toujours significative. Par la méthode AO on n'a jamais obtenu un résultat correct positif de

diagnostic de cancer supérieur à 50 % des cas. De faux diagnostics positifs de cancer ont été relevés dans 1,7 % des cas examinés par la méthode SH, et dans 2,4 % respectivement 3,4 % des cas examinés par les méthodes de Papanicolaou et AO.

Pour déceler cytologiquement un maximum de malades atteints de cancer, à une période précoce de l'évolution de la maladie quand un diagnostic manqué a beaucoup d'importance, c'est la méthode SH qui offrait le meilleur résultat. Pour le diagnostic final de malignité dans les poumons et les bronches, quand le cyto-diagnostic servait de méthode complémentaire ou alternative, il valait mieux choisir le résultat combiné obtenu des deux méthodes SH et de Papanicolaou. De cette façon le nombre de faux résultats positifs pouvait être notablement diminué.

Plusieurs prélèvements de crachats recueillis à de différentes occasions augmentaient la précision de diagnostic de toutes les méthodes de coloration. Mais trois prélèvements étaient en général nécessaires pour obtenir un diagnostic optimum.

126 prélèvements de sécrétions bronchiales de 121 malades furent examinés cytologiquement par les deux méthodes SH et de Papanicolaou. 43 malades avaient un cancer primitif des bronches, et 4 avaient des métastases pulmonaires d'une tumeur maligne extrapulmonaire. 27 prélèvements de sécrétions bronchiales de 25 malades, dont 7 avaient un cancer primitif des bronches furent examinés par la méthode AO.

Après la coloration d'un frottis des sécrétions bronchiales par la méthode SH, les cellules normales exfoliées donnaient en général une réaction assez pâle tandis que les cellules malignes au point de vue cytologique, développaient une réaction marquante. Ces résultats correspondaient aux résultats obtenus de l'examen des crachats traités de la même façon. Des cellules malignes au point de vue cytologique furent identifiées dans 49 % des cas de cancer par la méthode SH et dans 44 % des cas par celle de Papanicolaou, ce qui correspond aux résultats obtenus de l'examen du premier prélèvement de crachat. Un malade sur sept atteint d'un cancer, fut diagnostiqué par la méthode AO. De faux diagnostics positifs de cancer furent relevés dans 1,6 % respectivement 2,5 % des cas traités par les méthodes SH et de Papanicolaou. La méthode AO ne donna aucune fausse réponse positive.

CONCLUSIONS FINALES

Les investigations montrent que des cellules exfoliées dans les crachats et dans les sécrétions bronchiales, la concentration des groupes SH proteiques est en général basse dans les cellules normales et élevée dans les cellules malignes. Ceci a pu être utilisé pour le cyto-diagnostic de cancer primitif des

bronches avec au moins la même précision que la méthode de Papanicolaou. Dans certaines circonstances la méthode SH était préférable aux méthodes conventionnelles. De toutes les méthodes essayées la technique fluorescence d'acridine-orange ne donna pas d'aussi bons résultats, une méthode que l'on doit pour cela employer avec précaution en cyto-diagnostic.

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Uppsala, November 1963.

Lars-Gösta Wiman

APPENDIX

TABLE 22. Patient material. Results of cytological examination of sputum and bronchial secretions using the sulphydryl(SH), acridine-orange(AO) and Papanicolaou methods correlated to bronchoscopy findings and histopathological diagnosis in 107 patients with primary bronchogenic carcinoma and in two patients with primary pulmonary sarcoma

Pat. No. (1)	Sex (2)	Sputum				Bronchial secretions			Bronchoscopy findings (9)	Histopathological diagnosis (10)
		SH-method (3)	AO-method (4)	Papanicolaou method (5)	SH-method (6)	AO-method (7)	Papanicolaou method (8)			
1	M 42	V V V I V	V III IV IV	V IV V II V	V	III	III	Stenosis of the left main bronchus, biopsy positive	Squamous cell carcinoma	
2	M 59	IV I V	I I III	II I V	II	III	IV	Stenosis of the right main bronchus, biopsy positive		
3	M 72	III II I I III	--- I	I V I III III	IV	-	IV	Stenosis of the left main bronchus, biopsy positive		
4	M 53	III I V IV III (op.) I V II	--- (op.) - V I	I I I III (op.) I V II	I V (op.)	- V	I V	1. Abundant secretions in the right upper lobe bronchus, but no visible tumor. 2. Stenosis of the right upper lobe bronchus, biopsy negative.		
5	M 63	III IV III (op.) I V I III I V	--- (op.) --- I II	I III I (op.) I III I I V	III	-	I	Normal		
6	M 59	V V IV I	V V IV I	V V	I	I	I	Obturator tumor of the left upper lobe bronchus, biopsy suspected of cancer		
7	M 53	III	I	III	III	I	III	Normal		
8	M 63	III II I I I II III V V V	--- I I I	III II I I II III V V V	V	-	V	1. Reddened mucosa, no visible tumor, biopsy not performed. 2. Abundant secretions, no visible tumor, biopsy not performed		

Squamous cell carcinoma

upper lobe bronchus, biopsy im-
possible
Normal

10	M 58	I V I II I - - - - - II - - - V	-	I III I I I I III II I I III V	III	-	I
11	M 61	V IV IV V IV I	-	V IV IV V III II	I	-	V
12	F 61	V IV	-	II I	II	-	I
13	M 64	III IV	-	III V	V	-	III
14	M 70	III IV	-	IV V	IV II	-	III II
15	M 72	V III	-	V V	III	-	III
16	M 57	III V	-	V V	II	-	III
17	M 58	V V	-	V III	III	-	I
18	M 72	III II V III V V (op.) I I I	-	III V V II III II (op.) I I I	IV	-	II
19	M 54	V	-	IV	V	-	V

Squamous cell carcinoma

Table 22 (continued)

Pat. No. (1)	Sex (2)	Sputum			Bronchial secretions			Bronchoscopy findings (9)	Histopathological diagnosis (10)
		SH-method (3)	AO-method (4)	Papanicolaou method (5)	SH-method (6)	AO-method (7)	Papanicolaou method (8)		
20	M 68	V	-	IV	V	-	IV	Normal; blind biopsy suspected of cancer	Squamous cell carcinoma
21	M 56	III IV	-	I II	I	-	I	Stenosis of the right upper lobe bronchus, biopsy positive	
22	M 60	III IV IV IV V IV -	-	II IV IV V V V II V I I	II	-	III	Stenosis of the right upper lobe bronchus, biopsy negative	
23	M 62	IV I II II II V V V V	-	I I I III III V V IV V	III	-	II	No tumor visible, but abundant secretions in the right upper lobe bronchus, biopsy not performed	Adenocarcinoma
24	M 41	IV V IV V	-	V V V V V	V	-	V	Compression of the left main bronchus, biopsy not performed	
25	F 74	V	-	I	III	-	V	Obturator tumor of the right main bronchus, biopsy negative	Adenocarcinoma
26	F 71	I I III I	-	I I V I	V	-	V	Normal	Adenocarcinoma
27	M 47	V V III V II IV IV IV V	II I I I II I I II I	III I II II III II V V III	I	II	II	Stenosis of the right main bronchus, biopsy positive	Anaplastic carcinoma of the "oat cell" type
28	M 49	III II	I II	III III	I I	II II	III II	1. Polypoid tumor in the left upper lobe bronchus, biopsy negative. 2. Stenosis, biopsy positive	Low differentiated carcinoma
29	M 50	I III I I II V V	-	I II I III II III III	V	-	IV	Increasing stenosis of the right upper lobe bronchus, biopsy three times negative	Undifferentiated carcinoma
30	M 61	IV III I V	-	III III I IV	III	-	I	No visible tumor, but abundant secretions in left lower lobe bronchus	Anaplastic carcinoma of the "oat cell" type

Table 22 (continued)

Pat. No. (1)	Sex (2)	Sputum			Bronchial secretions			Bronchoscopy findings (9)	Histopathological diagnosis (10)
		SH-method (3)	AO-method (4)	Papanicolaou method (5)	SH-method (6)	AO-method (7)	Papanicolaou method (8)		
44	M 67	IV IV	II III	V V	-	-	-	Small tumor in the right upper lobe bronchus, biopsy positive	Squamous cell carcinoma
45	M 58	V	IV	V	-	-	-	Obturator tumor in the right main bronchus, biopsy positive	Squamous cell carcinoma
46	M 65	V V	I III	V III	-	-	-	Small tumor in the left lower lobe bronchus, biopsy positive	Squamous cell carcinoma
47	M 38	I I	I I	I I	-	-	-	Stenosis of the right main bronchus, biopsy positive	Anaplastic carcinoma of the "oat cell" type
48	M 67	III IV IV I	--- I	IV V IV I	-	-	-	Broadening of the carina but no visible tumor, biopsy not performed	Squamous cell carcinoma
49	M 52	III III V	- I	V I V	-	-	-	1. Normal. 2. Granulation tissue in the right lower lobe bronchus, biopsy positive	
50	M 73	I V V IV IV V	I I I I I I	I I I I I I	-	-	-	Bronchoscopy not performed	
51	M 66	I III	-	I V	-	-	-	Obturator tumor in the left main bronchus, biopsy positive	Squamous cell carcinoma
52	M 64	V	-	V	-	-	-	1. Normal, biopsy not performed. 2. Compression of the left main bronchus, biopsy negative. 3. Obturating tumor in the left main bronchus, biopsy positive	
53	M 62	II III	-	I IV	-	-	-	Stenosis of a left lower lobe bronchus, biopsy positive	
54	M 64	V	-	V	-	-	-	Stenosis of a right lower lobe bronchus, biopsy positive	Squamous cell carcinoma
55	F 67	I V I	-	III I IV	-	-	-	1. Normal, blind biopsy negative. 2. Obturating tumor in the right	

56	M 64	III	-	IV	-	-	-	Normal
57	M 67	II III IV III	-	III III III	-	-	-	Redness and pus in the left upper lobe bronchus, biopsy not performed
58	M 60	V	-	I	-	-	-	Normal
59	M 75	IV	-	V	-	-	-	Result of bronchoscopy unknown
60	M 65	III	-	I	-	-	-	Tumor in the right lower lobe bronchus, biopsy positive
61	M 65	I V I	-	I V I	-	-	-	Tumor in the left main bronchus, biopsy positive
62	M 52	V V V	-	V V V	-	-	-	Obturator tumor in the right main bronchus, biopsy positive
63	M 47	III	-	I	-	-	-	1. Normal. 2. Obturator tumor of left lower lobe bronchus, biopsy positive
64	M 61	IV I I I I	-	II I III I	-	-	-	No visible tumor, but reddened mucosa; biopsy not performed
65	F 71	II	-	III	-	-	-	Bronchoscopy not performed
66	M 60	IV V V	II V V	III V V	-	-	-	Stenosis of the right main bronchus, biopsy positive
67	M 62	I I	I I	I I	-	-	-	Bronchoscopy not performed
68	M 85	I	I	I	-	-	-	Bronchoscopy not performed
69	M 60	V I III III	IV I I I	IV I I	-	-	-	Thickened mucosa of left lower lobe bronchus, biopsy negative
70	M 74	I	I	I	-	-	-	Normal
71	M 61	IV I V IV I V	-	II III I I V	-	-	-	Stenosis of the right upper lobe bronchus, biopsy negative
72	F 51	I	-	V	-	-	-	Bronchoscopy not performed
73	F 72	V V	-	V III	-	-	-	Diffuse bronchitis in the left main bronchus, biopsy not performed
74	F 74	IV V V	-	III V V	-	-	-	Diffuse bronchitis in the left main bronchus, biopsy not performed

Squamous cell carcinoma

Adenocarcinoma

Table 22 (continued)

Pat. No. (1)	Sex Age (2)	Sputum				Bronchial secretions			Bronchoscopy findings (9)	Histopathological diagnosis (10)
		SH- method (3)	AO- method (4)	Papanicolaou method (5)	SH- method (6)	AO- method (7)	Papanicolaou method (8)			
75	F 61	III	-	V	-	-	-	Obturation of the left main bronchus, biopsy suspected of cancer	Adenocarcinoma	Undifferentiated carcinoma
76	F 66	V V IV V	-	III V IV V	-	-	Normal			
77	M 67	V	-	IV	-	-	Normal			
78	F 52	V	-	V	-	-	Blood and abundant secretions in the left upper lobe bronchus, biopsy impossible			
79	M 64	I I	-	I I	-	-	Tumor in the right upper lobe bronchus, biopsy negative			
80	F 64	V V	-	V V	-	-	Bronchoscopy not performed			
81	F 68	V	-	IV	-	-				
82	M 64	V II V III V IV	-	V V III V V IV	-	-				
83	M 80	I II III	-	I III IV	-	-				
84	M 73	V	-	III	-	-				
85	M 62	III	-	IV	-	-	Stenosis of a left lower lobe bronchus, biopsy positive	Undifferentiated carcinoma		
86	M 68	V III	-	V III	-	-	Bleeding from a left lower lobe bronchus, biopsy negative			
87	M 65	IV V	-	III V	-	-	Visible stenosis of the left upper lobe bronchus, biopsy negative			
88	M 63	III I I I	-	IV III I V	-	-	Normal	Low differentiated carcinoma in metastasis of a left side rib	Undifferentiated carcinoma	
		I I								

Table 22 (continued)

Pat. No. (1)	Sex (2)	Sputum			Bronchial secretions			Bronchoscopy findings (9)	Histopathological diagnosis (10)
		SH- method (3)	AO- method (4)	Papanico- laou method (5)	SH- method (6)	AO- method (7)	Papanico- laou method (8)		
106	M 79	V	-	V	-	-	-	Bronchoscopy not performed	Clinical diagnosis of cancer
107	F 88	II II	-	II IV	-	-	-	Bronchoscopy not performed	Clinical diagnosis of alveolar cell carcinoma (bronchio- lar carcinoma)
108	M 46	V	-	II	-	-	-	Diffuse bronchitis, no biopsy	Primary pulmonary sarco- ma
109	M 57	III	-	III	-	-	-	Tumor visible in the right lower lobe bronchus, biopsy impossible	Rhabdomyosarcoma, pro- bably primary of the lung

TABLE 23. Results of cytological examination of sputum and bronchial secretions using the sulphydryl (SH), arctane orange (AO) and Papanicolaou methods correlated to bronchoscopy findings and histopathological diagnosis in 11 patients with pulmonary metastases from malignant tumors of extrapulmonary origin

Pat. No.	Sex	Sputum				Bronchial secretions			Bronchoscopy findings (9)	Histopathological diagnosis (10)
		SH- method (3)	AO- method (4)	Papanico- laou method (5)	SH- method (6)	AO- method (7)	Papanico- laou method (8)			
110	M 50	V V V V V V V V	V V V V V V V V	V V V V V IV V V	V	V	V	Normal	Adenocarcinoma of the rectum with pulmonary carcinosis	
111	F 68	IV	-	IV	V	-	II	Small tumor of the right main bronchus, biopsy positive	Hypernephroma with pul- monary metastases	
112	M 68	I I IV I	-	I I IV I	I	-	I	Normal	Hypernephroma with pul- monary metastases	
113	M 50	II IV II V -	-	I II III V I	II	-	I	Normal	Hypernephroma with pul- monary metastases	
114	M 73	V -	- - - V	V V V V	-	-	-	Reddened mucosa and abundant secretions in the left main bron- chus, biopsy not performed	Carcinoma of the papilla of Vater and pulmonary me- tastases	
115	M 68	V V V IV V	-	III V V V V	-	-	-	Normal	Carcinoma of the pancreas	
116	F 49	III I	-	III III	-	-	-		Gastric ulcer with malig- nant changes and pulmo- nary metastases of adeno- carcinomatous type	
117	M 68	I I I	-	II II II	-	-	-		Carcinoma of the ileum, colon and rectum with pulmonary metastases	
118	F 54	IV V III	-	III II IV	-	-	-	Bronchoscopy not performed	Carcinoma of the left breast and pulmonary metastases	
119	F 50	II	-	II	-	-	-		Liposarcoma of the left leg with pulmonary metastasis, suspicious of sarcoma in aspiration biopsy.	
120	M 66	I	-	I	-	-	-		Clinical diagnosis of hyper- nephroma with pulmonary metastases	

TABLE 24. Results of cytological examination of sputum and bronchial secretions using the sulphydryl(SH), acridine orange(AO) and Papanicolaou methods correlated to bronchoscopy findings and clinical or histopathological diagnosis in five patients with malignant systemic diseases in and five patients with benign neoplastic pulmonary diseases

Pat. No. (1)	Sex (2)	Sputum			Bronchial secretions			Bronchoscopy findings (9)	Histopathological diagnosis (10)
		SH-method (3)	AO-method (4)	Papanicolaou method (5)	SH-method (6)	AO-method (7)	Papanicolaou method (8)		
121	F 50	I V V I III	-	II IV V II IV	-	-	-		Myeloid leukemia with pulmonary leukemic infiltrations
122	M 79	I	-	I	-	-	-	Bronchoscopy not performed	Chronic lymphatic leukemia
123	F 56	I I IV III I	-	I I I II	-	-	-		Malignant lymphogranulomatosis
124	M 72	I I I I	-	II III I	-	-	-		Myelomatosis
125	M 65	I I	-	I I	-	-	-	Granulation tissue and squamous metaplasia Normal	Mycosis fungoides
126	F 52	I	-	I	II	-	II		Pulmonary hamartoma
127	F 47	III I	I I	III I	-	-	-	Normal	Pulmonary carcinoid
128	M 57	II	-	II	I	-	I		Clinical diagnosis of benign pulmonary neoplasm
129	F 67	I	-	I	-	-	-	Normal	Clinical diagnosis of benign pulmonary neoplasm
130	F 72	II II	-	II II	-	-	-	Bronchoscopy not performed	Clinical diagnosis of benign pulmonary neoplasm

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